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ON THE RELATIONS BETWEEN ATMOSPHERIC POLLUTION IN URBAN AND RURAL LOCALITIES AND MORTALITY FROM CANCER, BRONCHITIS AND PNEUMONIA, WITH PARTICULAR REFERENCE TO 3 : 4 BENZOPYRENE, BERYLLIUM, MOLYBDENUM, VANADIUM AND ARSENIC

PERCY STOCKS

From Colwyn Bay, North Wales

Received for publication July 12, 1960

History and purpose of study

In 1954 arrangements were made jointly with the British Empire Cancer Campaign, Medical Research Council and Department of Scientific and Industrial Research to commence an investigation into the amounts of smoke, certain polycyclic hydrocarbons and trace elements in the atmosphere at a number of places in North Wales and Liverpool Hospital Region with a view to relating them with the mortality from Cancer in the areas concerned. In the first instance 10 instruments for continuous collection of smoke on filter papers were placed in urban and rural situations. These were equipped with meters to measure the volume of air passing through, and the filter papers were changed at weekly or monthly intervals and sent for hydrocarbon analysis to the M.R.C. Group at St. Bartholomew's Hospital, London, and for trace element analysis to the Fuel Research Station of the D.S.I.R. at East Greenwich. Results of the measurements of 3 : 4 benzopyrene, pyrene, 1 : 12 benzoperylene and fluoranthene during 6 months from October, 1954, were published in 1955 (Stocks and Campbell, 1955) and results for two years together with data concerning trace elements during 1955 were given in 1958 (Stocks, 1958*a*).

In the autumn of 1956 filters were installed at 13 more places in North Wales, Liverpool and Lancashire, including two in the Mersey Tunnel, two in bus and motor garages, and one in an office, each with an outside control. Hydrocarbon analyses for these and the subsequent series were made by Mr. B. T. Commins of the M.R.C. Group at the Dunn Laboratory. The filters were accompanied by high-speed instruments working alongside the low-speed type to obtain quantities of smoke more adequate for the spectrographic study of trace elements. A report on the smoke and hydrocarbon data from the 23 stations from October 1956 to April 1957 was published with tentative correlations with standardised mortality indices for cancer of the lung and intestine in 15 areas (Stocks, 1958*b*) At the end of 1957, aided by a grant from the Medical Research Council, the mortality data were extended to include bronchitis and pneumonia, and the region was widened by installing low-speed and high-speed filters at five more

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places in Lancashire (including one in a smokeless zone), at six places in the West Riding of Yorkshire and at four sites in a large steelworks, and of low-speed filters at three places in Tyneside conurbation. This made a total of 41 filter locations as specified in Table I.

TABLE I.—*Localisation of* 41 *Instruments to Collect Smoke Samples for Analysis.*

County or conurbation	Representative locations	Alternative sites in conurbations	Special locations
Anglesey	Llangefni	—	—
Caernarvon	Conway Valley	—	—
Merioneth*	Blaenau Ffestiniog*	—	—
Denbighshire	Ruthin	—	—
	Wrexham	—	—
Flintshire	Flint	—	4 in steelworks.†
Merseyside conurbation	Birkenhead	Seaside (Hoylake)	2 in Mersey tunnel.‡
	Bootle	Riverside‡	Bus depot (Edge Lane).‡
	Liverpool, Princes Road	Roe Street‡ Edge Lane‡	Motor car garage.‡ Office interior.*
Cheshire	Chester	—	—
	Tattenhall	—	—
S.E. Lancashire conurbation	Salford, Regent Road	Smokeless zone‡	—
Lancashire	St. Helens	—	—
	Warrington‡	—	—
	Ormskirk	—	—
	Darwen‡	—	—
	Burnley‡	—	—
	Lancaster‡	—	—
West Yorkshire conurbation	Leeds‡	—	—
	Keighley‡	—	—
	Elland‡	—	—
West Riding	York‡	—	—
	Wetherby‡	—	—
	Ripon‡	—	—
Tynside conurbation	Gateshead‡*	—	—
	Newcastle-on-Tyne‡ (Wharncliffe St.)*	Shields Road‡*	—

* No trace element analyses. † No hydrocarbon analyses.
‡ Analyses of 7 hydrocarbons were made here, and of 4 hydrocarbons elsewhere.

The present paper deals with the representative locations, which alone can be related to mortality rates. Hoylake has not been included with these but considered as a special Merseyside site because many of the residents travel to Liverpool or Birkenhead to work or have retired after working there, and their death rates cannot be fairly related to the local atmospheric conditions. The results from the alternative and special sites have been reported upon in a separate paper (Stocks, Commins and Aubrey, 1960). Data of total smoke and 4 polycyclic hydrocarbons are available at each of the 26 places in the first column of Table I, and amounts of trace elements at 23 of them.

A previous study was made also of the amounts of undissolved deposit and of smoke in those county boroughs of England and Wales and administrative areas of Lancashire and the West Riding of Yorkshire where measurements had been recorded by the D.S.I.R., relating these with standardized mortality ratios for cancer and bronchitis (Stocks, 1959). This has now been extended by addition of more towns, using a more adequate social index, and a similar study has been made of Greater London.

The main purposes of this paper are (1) to establish more firmly the association between smoke and mortality from lung cancer and respiratory disease, and (2) to ascertain which of the polycyclic hydrocarbons and which of the trace elements are concerned in this.

Evaluation of smoke and polycyclic hydrocarbons in air

The amount of smoke per cubic metre of air is estimated from a direct photometric measure of the blackness of the circular portion of known area of the filter paper through which a measured volume of air has been drawn by a pump operating continuously for a suitable period. At the end of the period, which may be a week in a highly urbanised district or a month in a rural district, the paper is changed and the results from all the filter papers throughout a year are aggregated when an annual average is required. The blackness readings are converted to milligrams of smoke by means of a standard calibration curve. The calibration was originally carried out on London smoke and it has been found to be reasonably satisfactory for smoke in other large towns in Great Britain. Persistent errors in the absolute values of the smoke concentration determined in this way would not affect comparisons between districts, but there may be local variations in the " blackness " per unit weight of smoke. The standard calibration applies to the usual mixture of domestic smoke plus a little industrial and traffic smoke found in the general atmosphere of British towns and it is unlikely to hold good for special locations where industrial or traffic smoke predominate, such as road tunnels. Any variations of this kind would be liable to reduce the correlation coefficients between smoke and mortality.

The polycyclic hydrocarbons are determined quantitatively and a direct measure of their concentration in the air is obtained by dividing by the volume of air sampled in each case. They can also be expressed as a proportion of the total amount of suspended matter (smoke) as in Table III, and this may be of some importance, quite apart from the absolute concentration in air, but such figures are subject to the errors mentioned above which affect the denominators of the proportions. Sources of error which might depress correlations between amounts of hydrocarbons and mortality rates or social factors include analytical errors, instability of some of the substances leading to partial loss by complex processes such as oxidation or evaporation during the interval between collection of a sample and its analysis, and faults in obtaining representative samples by the choice of sites for the filters. Concerning the analytical technique a modification of this was introduced (Commins, 1958) and applied in 1958–59, but all data used in this paper from previous years have been corrected to allow for the effects of this. As to stability, pyrene and fluoranthene are less stable than 3 : 4 benzopyrene and 1 : 12 benzoperylene and if changes occurred more rapidly in samples from some areas than in samples from elsewhere, correlations between amounts of these substances and mortality rates might be depressed rather more than was the case for the other hydrocarbons. Some internal evidence on this point is provided by the inter-correlations between recorded amounts of the 4 hydrocarbons in the air of 26 localities at the head of Table VI. The first two, 3 : 4 benzopyrene and 1 : 12 benzoperylene, are closely related (0·956), but the last two, pyrene and fluoranthene, are just as closely related (0·951), and the average correlation between pyrene and the other 3 substances is 0·923 compared with 0·929 for the more stable benzopyrene. It appears that the rate of change

of pyrene in the samples must have been so uniform as not to affect correlations with other factors appreciably.

Validity of correlations between smoke measurements and mortality rates

A source of difficulty in attempts to correlate these factors is that neither is constant in time, and exposure to air pollution for a number of years may be necessary to produce serious respiratory disease or initiate cancer. In order to obtain meaningful rates from specific diseases in separate districts deaths through a period of years have to be aggregated and the rates standardized to allow for differing age distributions in the populations concerned. Changes in the international rules for classifying deaths to their underlying cause from 1950 onwards, and changes in the rules for residence allocation of persons dying from chronic diseases after a long time in hospital which took effect in 1954, made it advisable to use the period 1950–54 for lung cancer and 1950–53 for the other disease groups in this study. The smoke measurements were made for the most part during 1955–58. It has to be assumed, therefore, that although the amount and nature of air pollution by smoke may have changed in the last 10 or 20 years, the *relative* changes have been the same in all the areas concerned, in which case the correlations with other factors would not be affected. Departures from this assumed constancy of relative change would tend almost certainly to reduce the true correlations and the observed coefficients can safely be regarded as minimal figures. This difficulty must arise in all attempts to relate mortality from chronic diseases with measures of air pollution of which records exist only for recent years, and action which has now started to reduce such pollution in the worst areas makes it an urgent matter to make such epidemiological studies before the opportunity has gone.

Another possibly important source of error was the reliance on a single instrument site to measure the average air conditions to which the population of the district surrounding it was exposed in the course of a year. A study of this problem has been made in Sheffield, where it was found that when any 8 instruments were chosen to assess the average smoke concentration to which the whole of the population in the city was being exposed, the mean of the 8 had a coefficient of variation of about 10 per cent (Clifton *et al.*, 1959). This variation increased in the inverse ratio of the square root of the number of filter sites, reaching about 28 per cent for a single site. Sheffield is peculiar in having within its area of 62 square miles altitudes above sea level ranging from 100 to 1400 feet and densities of population in separate wards ranging from 2 to 62 per acre, and the coefficient of variation is undoubtedly higher than would be found in most cities. In Liverpool where the river is a disturbing factor, smoke concentrations at three locations were 179, 215 and 204 in the spring-summer half and 500, 624, 604 in autumn-winter, measured in mg./1000 m^3. In small towns variation would generally be less, and in the 26 areas used in the present study the coefficient of variation for a single site can reasonably be taken as 20 per cent.

In order to find what effect such errors could have on the correlation coefficients with mortality several tests were made on the lung cancer figures, assuming the root mean square error of the smoke readings to be 20 per cent of the true averages in the area. The 26 ratios (F) between the true average smoke value in a district and the smoke reading at a single location in it were presumed to have a normal distribution with standard deviation 0·20 and they were first arranged in

order according to a table of random numbers and applied as correcting factors to the observed smoke figures in the areas when arranged in alphabetical order of place names. The resulting correlation coefficient between the corrected smoke values and lung cancer rates was 0·77, and when the factors in the same random order were applied to the areas arranged in order of their lung cancer rates the result was 0·82. It is to be expected, however, that the magnitude of the error, regardless of its sign, would in general increase with the size of the district providing the death rates, and the districts were therefore arranged in ascending order of their size and the presumed errors ($F - 1$) were also arranged by magnitude with + and − errors alternating, each smoke reading being then corrected by the corresponding factor (F) to estimate the true average for the district. This led to a correlation coefficient with lung cancer of 0·873, the same as the original uncorrected value (Table IV). The conclusion from these tests is that the errors arising from the use of only a single instrument site in each district have not affected the correlation coefficients sufficiently to invalidate the deductions made from such data in this paper. For greater accuracy it would be advisable in future stuides of this kind to instal, if practicable, more than one instrument in districts with population (P) exceeding 50,000. A convenient criterion for the number (n) to be used would be $n = \sqrt{P}/20{,}000$, the choice of sites being related to the population distribution within the area as suggested by the Sheffield study (Clifton *et al.*, 1959).

Social environment

Several measures of the average social conditions in administrative areas have been used by statisticians in the study of death rates. The most readly available one is the density of population per acre, and in international comparisons of towns this may be the only one which can be obtained from official data. Death rates from respiratory diseases are highly correlated with this measure of social conditions, but only in recent years has it been realised that the main reason for this is the simple fact that population per acre is in itself a measure, in Britain at least, of the number of domestic chimneys per acre and therefore of the average density of domestic smoke in the atmosphere of the area. There are other factors contributing to the correlations, for example districts with high densities of population tend to have more heavy industries, more unskilled workers and poorer housing conditions. That these other factors are of minor importance compared with smoke in producing the correlation of 0·839 in the 26 northern areas between lung cancer mortality of males and persons per acre (Table IV) is evident from the fact that this figure is reduced to 0·327 when smoke density is held constant. Population per acre is not, therefore, a good measure of non-atmospheric social conditions since it is so closely linked with smoke density that to hold it constant for 2nd order correlations over-corrects for the social factors. It was used in a previous study of towns (Stocks, 1959) only because at the time of preparing the paper no better index was available, but this erred on the safe side by reducing lung cancer and bronchitis correlations with smoke too severely.

Other social indices which have been used by the Registrar General in the past are the housing indices based on numbers of persons per room and the proportion of occupied and retired males in unskilled occupations. In the present paper two measures of the last mentioned kind have been used. After the census of 1931 the proportion of males aged 14 and over in social classes IV and V was

employed as a " social index " for large towns (Registrar General, 1936), and that index has been used here in the study of smoke in county boroughs, and a similar index based on 1951 census has been used for 40 districts of Greater London (Tables XI, XIII and XV). Where rural areas or small country towns occur in the survey, social class IV may include large numbers of agricultural labourers whose mortality rates are much below the class average, and the proportion in class V alone is a better social index. For the 26 northern districts this latter index has been used (Tables III, IV, V, X), and also for the Lancashire and Yorkshire districts but in this case as an alternative the rural districts have been omitted and the index based on classes IV–V applied (Tables XII and XIV). In Greater London the correlations between smoke concentration and 4 social indices were found to be : persons per acre 0·776, persons per room 0·447, class IV–V index 0·382, class V index 0·368. In the Lancashire and Yorkshire districts the coefficient with persons per acre was 0·611, and when rural areas were omitted the coefficient with class IV–V index was 0·397.

Smoke, polycyclic hydrocarbons and mortality in 26 areas

In Table II the 26 filter locations which were chosen with a view to measuring average atmospheric conditions in the districts around them are arranged in order of the mean smoke concentrations during a complete year, ranging from 15 mg. per 1000 cubic metres of air in Conway Valley to 562 in Salford. The next 4 columns show the amounts of 3 : 4 benzopyrene, 1 : 12 benzoperylene, pyrene and fluoranthene in parts per million of the smoke, and the last 4 columns show the same hydrocarbons in micrograms per 1000 cubic metres of air. The hydrocarbon rates are based on the improved analytical methods introduced in 1958 and consequently some are not comparable with rates published previously, but all figures in the table are comparable one with another.

The geographical variation in amounts of the hydrocarbons per unit of smoke is less than that shown by the concentrations in air ; for the former the coefficients of variation of the 4 substances are 35, 39, 37 and 52, and for the latter they are 81, 72, 77 and 71. Irritant effects on the respiratory tract might depend on the number of particles of a particular size and containing a harmful substance, on the average amount of it present in a particle or simply on the total quantity of the substance inhaled regardless of its distribution in the particles. Since workers in very high concentrations of coal combustion products in gas works suffer only moderate irritant effects, the nature of the particles in domestic coal smoke must be important, but in comparing effects of ordinary atmospheres polluted by different amounts of domestic smoke it is to be expected that the particles collected by the standard filters will be similar in nature and that pathological effects will be most closely related to the total amounts of particular chemical substances inhaled, that is to say their concentration per unit volume of air. Table II shows that the annual average concentration of 3 : 4 benzopyrene ranged from 1 μg./1000 m^3 in the purest air to 108 in the most polluted ; for 1 : 12 benzoperylene it ranged from 0·4 to 74, for pyrene from 1 to 38 and for fluoranthene from 3 to 58.

Table III gives the population densities, social indices and standardised mortality ratios for cancer of the lung (males), bronchitis and pneumonia in the administrative areas where the 26 filters were located. Rates for stomach and breast cancer are not shown from considerations of space, but the S.M.R.'s for

TABLE II.—*Average Annual Amounts of Smoke and 4 Polycyclic Hydrocarbons in 26 Localities of Northern England and Wales, 1957 or 1958.*

Location of filter (ranked according to smoke density)	Year	Smoke (mg./1000 cu. m.)	Hydrocarbons (μg. per g. of smoke) 3:4 Benzopyrene	1:12 Benzoperylene	Pyrene	Fluoranthene	Hydrocarbons (μg. per 1000 cu. m. of air) 3:4 Benzopyrene	1:12 Benzoperylene	Pyrene	Fluoranthene
Conway Valley	7	15	75	37	81	263	0·9	0·4	0·8	3·3
Llangefni	7	53	198	82	118	169	6·8	3·1	5·0	4·6
Tattenhall	7	85	65	59	61	112	5·1	4·5	4·8	9·2
Wetherby	8	98	115	114	69	105	11·0	11·0	7·0	10·0
Ruthin	7	105	73	89	36	62	8·1	9·8	3·4	5·4
Blaenau Ffestiniog	7	124	69	95	81	187	5·5	21·0	12·0	20·0
Ripon	8	125	109	89	48	72	15·0	12·0	6·0	10·0
Elland	8	150	108	96	32	49	15·0	14·0	5·0	7·0
Flint	7	169	153	129	57	93	25·1	20·8	12·8	15·8
York	8	177	137	121	78	110	24·0	21·0	14·0	20·0
Chester	7	208	110	117	56	75	19·6	12·6	10·5	13·4
Ormskirk	7	226	123	136	45	66	26·1	28·0	10·7	16·1
Wrexham	7	264	128	96	60	60	24·9	24·7	12·0	15·2
Lancaster	8	276	71	49	34	51	20·0	14·0	9·0	14·0
Darwen	8	288	125	123	60	85	36·0	35·0	17·0	25·0
Keighley	8	291	109	108	34	61	32·0	32·0	10·0	18·0
Burnley	8	311	105	95	14	24	32·0	30·0	4·0	8·0
Birkenhead	7	336	169	153	71	89	47·1	42·3	20·7	23·7
Bootle	7	362	152	150	68	97	51·5	49·4	24·9	33·2
St. Helens	7	376	205	151	71	105	69·2	52·1	28·0	44·2
Warrington	7	380	123	98	34	64	48·8	41·0	14·3	28·3
Gateshead	8	399	156	125	73	101	62·0	50·0	29·0	40·0
Leeds	8	421	100	95	34	49	42·0	40·0	14·0	21·0
Liverpool (Princes Rd.)	7	455	241	182	66	108	99·9	74·2	29·8	47·9
Newcastle-on-Tyne*	8	505	149	145	74	115	75·0	73·0	37·0	58·0
Salford (Regent Rd.)	8	562	189	108	66	87	108·0	62·0	38·0	49·0
Mean	—	260	129·0	109·3	58·5	94·6	35·0	29·9	14·6	21·5

* Wharncliffe Street.

other cancer are given. The numbers of deaths of females from lung cancer in many of the areas were too small for reliable conclusions to be reached. All the S.M.R.'s are based on England and Wales as a whole taken as 100, and they range from 23 to 165 for lung cancer, 68 to 122 for other cancer, 18 to 259 for bronchitis and 61 to 227 for pneumonia.

A social factor which is related indirectly with density of population and directly with lung cancer incidence is the amount of cigarette smoking, and it is not possible to correct for this since complete data are lacking, but its importance in the North Wales and Merseyside region has been measured by actual enquiries into tobacco smoking histories of hospital patients. Lung cancer mortality in heavy smokers was found to differ little in urban and rural areas, in medium cigarette smokers the urban/rural ratio was about 1½ and in light smokers about 2½ (Stocks, 1958*a*). The effect of actually correcting for differences in smoking

TABLE III.—*Standardised Mortality Ratios for Cancer, Bronchitis and Pneumonia in 26 Areas of Northern England and Wales where Smoke Analyses have been made.*

Administrative area and county‡		Persons per acre	Social class (V) index	S.M.R.'r in 1950–53* Lung cancer (M.)	Other sites of cancer† (M.)	(F.)	Bronchitis (M.)	(F.)	Peumonia (M.)	(F.)
Nant Conway R.D.	CA	0·1	109	59	81	88	29	91	94	181
Llangefni U.D. (1)	A	0·2	105	54	110	104	18	48	126	100
Tarvin R.D. (2)	CH	0·2	107	59	68	97	55	57	61	76
Wetherby R.D.	Y	0·3	94	64	88	89	59	42	102	101
Ruthin M.B. and R.D.	DE	0·1	74	23	84	93	28	75	82	90
Ffestiniog U.D.	M	0·4	61	74	98	154	46	12	154	143
Ripon U.D. and R.D.	Y	0·2	149	72	84	93	66	88	146	107
Elland U.D.	Y	3·2	99	70	81	106	79	59	114	123
Flint M.B.	F	2·1	238	80	112	144	50	105	118	40
York C.B.	Y	16·4	162	92	113	106	95	75	120	113
Chester C.B.	CH	11·6	156	115	98	97	113	81	93	102
Ormskirk U.D.	L	1·3	142	92	92	110	82	97	109	86
Wrexham M.B.	DE	10·6	141	76	118	99	82	68	125	90
Lancaster M.B.	L	5·0	199	95	91	108	81	76	117	93
Darwen U.D.	L	5·2	211	81	104	109	112	136	91	125
Keighley M.B.	Y	2·4	116	76	104	119	116	167	91	92
Burnley C.B.	L	18·1	156	92	90	99	139	181	134	117
Birkenhead C.B.	CH	16·6	222	137	117	103	125	116	180	141
Bootle C.B.	L	13·1	295	143	85	92	194	164	214	219
St. Helens C.B.	L	13·9	227	111	102	109	160	141	144	165
Warrington C.B.	L	18·3	246	125	118	96	199	223	132	154
Gateshead C.B.	DU	25·7	192	113	113	109	136	111	164	119
Leeds C.B.	Y	13·2	135	134	104	99	162	141	200	202
Liverpool C.B.	L	28·9	245	160	119	103	139	138	227	245
Newcastle-on-Tyne	N	26·3	166	123	118	103	114	113	161	126
Salford C.B.	L	34·2	209	165	122	112	259	240	176	154

Notes

(1) With Aethwy and Twrcelyn R.D's. (2) District surrounding Tattenhall. * 1950–54 for lung cancer. † All sites except lung, stomach, breast. ‡ A, Anglesey; CA, Caernarvon; CH, Cheshire; DE, Denbigh; DU, Durham; F, Flint; L, Lancashire; M, Merioneth; N, Northumberland; Y, Yorkshire West Riding.

habits between North Wales and Liverpool was to reduce the urban/rural ratio for lung cancer rates in men from 2·4 observed to 2·1, and for smaller towns the effect was less than this (Stocks, 1955). A sampling enquiry by the Tobacco Manufacturers' Standing Committee (1959) showed that the average daily consumpton of packeted cigarettes per adult in 1956 was 9 in rural districts, 9·6 in London and about 11 in other large towns. Applying these to the regression graphs of lung cancer mortality on number of cigarettes smoked in the same kind of area, the rate in large towns might be enhanced by about 20 per cent because of this difference. As a test of the effect this could have on the correlation between lung cancer rates and smoke density the S.M.R.'s in Table III were reduced to that extent for the 8 towns of over 100,000 population and the resulting

coefficient was 0·72 instead of 0·87, the uncorrected figure. The coefficients of 0·52 in 30 county boroughs (Table XIII) and 0·53 in 42 Lancashire and Yorkshire urban areas (Table XIV) are unlikely to be affected appreciably by differences in smoking habits.

TABLE IV.—*Correlations in 26 Localities of Northern England and Wales between Smoke and Hydrocarbons in Air and Standardised Mortality from Lung Cancer in Males in* 1950–54, *Population Density and Social (Class V) Index.*

	1st order coefficients with				2nd order with lung cancer, with constant		
	Lung cancer	Smoke	Persons per acre	Social index	P.P.A.	Index	Smoke
	Smoke and hydrocarbons per cubic metre of air						
Smoke (total) . .	0·873	—	0·871	0·649	0·531	0·756	—
3 : 4 Benzopyrene .	0·862	0·927	0·895	0·662	0·457	0·738	0·288
1 : 12 Benzoperylene .	0·832	0·936	0·856	0·667	0·409	0·667	0·090
Pyrene . . .	0·751	0·848	0·798	0·635	0·250	0·543	0·045
Fluoranthene . .	0·779	0·876	0·821	0·619	0·293	0·622	0·020
Lung cancer . .	—	—	0·839	0·735	—	—	—
	Hydrocarbons per unit weight of suspended matter						
3 : 4 Benzopyrene .	0·587	—	0·604	0·591	0·185	0·272	—
1 : 12 Benzoperylene .	0·464	—	0·411	0·606	0·240	0·044	—
Pyrene . . .	0·004	—	0·073	−0·084	−0·109	0·096	—
Fluoranthene . .	−0·180	—	−0·248	−0·252	0·052	0·018	—

Correlations between polycyclic hydrocarbons and lung cancer rates

Table IV shows the correlaton coefficients between lung cancer mortality in men and the social and air-pollution indices, and partial coefficients when the social factors are constant. For the reasons already stated elimination of persons per acre reduces the primary coefficients unduly, but the figures are given for comparison with those corrected for proportions of men in social class V which are free from that objection. Concentrations of the hydrocarbons in air are highly correlated with lung cancer after eliminating the latter social factor, with odds exceeding 1000 to 1 against such coefficients arising fortuitously in the case of 3 of them. All coefficients exceeding 0·375 are significant at the conventional level of 1 in 20 probability. Amounts of the substances per unit of smoke show no significant associations with lung cancer, and only in the case of 3 : 4 benzopyrene is there any suggestion that this may be of any importance.

Table VI indicates in its first section that the hydrocarbon concentrations in air are very highly correlated one with another, owing to their common origin, and when a substance x is present in large amount the others must tend to be increased also in consequence. If x is concerned in disease incidence and the others are not, the correlations between the others and the disease rates should become inappreciable when the concentration of x is held constant. In effect this means that in 26 areas having the same degree of air pollution by the active substance x no correlations would be found between lung cancer mortality and the amounts of the other hydrocarbons present in the air. In the first column of the second section of the table the concentration of each hydrocarbon has been held constant in turn, with the following result for lung cancer. With 3 : 4 benzopyrene constant the total smoke coefficient is reduced from 0·873 (Table IV) to

0·389, indicating the probable presence of some other constituents of smoke which affect the lung, such as certain trace elements as will be seen later; the benzoperylene coefficient is reduced to 0·055, and the other two hydrocarbons cease to show any relation with lung cancer. With 1 : 12 benzoperylene held constant the 3 : 4 benzopyrene coefficient is still significant (0·394) whereas the others disappear; and the making of pyrene and fluoranthene constant scarcely affects the benzopyrene coefficient and leaves the benzoperylene figure still significant. As the figures at the foot of the first column indicate, pyrene and fluoranthene derive their statistical connections with lung cancer from their association with 3 : 4 benzopyrene in smoke; 1 : 12 benzoperylene may have an independent effect, but if so it is small in comparison with that of 3 : 4 benzopyrene which clearly emerges from this analysis as the hydrocarbon of outstanding importance. These results agree with experimental evidence about the carcinogenic potency of the hydrocarbons.

Correlations between polycyclic hydrocarbons and mortality from other diseases

Table V shows no appreciable correlations between amounts of smoke or any of the hydrocarbons in air and mortality from cancer of the stomach in either sex or cancer of the breast or other organs in females after correcting for social index. Other cancer in males, for which figures are not shown in the tables, gives primary coefficients of 0·64 with smoke, 3 : 4 benzopyrene and pyrene, 0·63 with 1 : 12 benzoperylene and 0·61 with fluoranthene, and when social index and other hydrocarbons are constant the benzopyrene and pyrene coefficients are still appreciable but not significant and no conclusion can be drawn. The group includes the larynx, oesophagus and intestine, of which separate data are not obtainable.

Bronchitis gives primary correlations similar to those for lung cancer, and the partial coefficients when social index is constant are significant for males in respect of each hydrocarbon and for females in respect of 3 : 4 benzopyrene and

TABLE V.—*Correlations in 26 Localities of Northern England and Wales between Smoke and Hydrocarbons in Air, Standardised Mortality from Cancer of the Stomach, Breast and Other Sites, Bronchitis and Pneumonia in* 1950–53, *and Social (Class V) Index.*

	Cancer (excluding lung)				Bronchitis		Pneumonia	
	Stomach		Breast	Other				
	(M.)	(F.)	(F.)	(F.)	(M.)	(F.)	(M.)	(F.)
	1st order coefficients							
Smoke (total)	0·241	0·308	0·104	−0·002	0·869	0·751	0·666	0·479
3 : 4 Benzopyrene	0·261	0·305	0·136	0·024	0·809	0·699	0·699	0·549
1 : 12 Benzoperylene	0·141	0·281	0·129	0·013	0·741	0·672	0·715	0·511
Pyrene	0·045	0·219	0·071	0·093	0·683	0·530	0·662	0·443
Fluoranthene	0·118	0·319	−0·022	0·101	0·700	0·451	0·636	0·583
	2nd order coefficients with social index constant							
Smoke (total)	0·123	0·077	—	—	0·768	0·570	0·491	0·256
3 : 4 Benzopyrene	0·146	0·073	—	—	0·693	0·513	0·540	0·356
1 : 12 Benzoperylene	*Nil*	0·048	—	—	0·532	0·430	0·565	0·297
Pyrene	,,	0·077	—	—	0·448	0·211	0·490	0·209
Fluoranthene	,,	0·140	—	—	0·489	0·094	0·455	0·418

1 : 12 benzoperylene. Table VI shows however that when 3 : 4 benzopyrene is held constant the other hydrocarbons cease to have any association with bronchitis in either sex, whereas the benzopyrene coefficients average 0·584 when the others are held constant. This points to 3 : 4 benzopyrene as the hydrocarbon concerned with bronchitis mortality.

Pneumonia shows in Table V significant correlations when social index is constant for males in respect of each hydrocarbon and for females in respect of

TABLE VI.—*Correlations with Amounts of Smoke and Hydrocarbons in Air of 26 Localities when the Effect of One Hydrocarbon on the Coefficient is Eliminated.*

Inter-correlations between Smoke and Hydrocarbons

	Smoke	Benzopyrene	Benzo-perylene	Pyrene	Fluoran-thene
3 : 4 Benzopyrene	0·927	—	0·956	0·915	0·917
1 : 12 Benzoperylene	0·936	0·956	—	0·902	0·927
Pyrene	0·848	0·915	0·902	—	0·951
Fluoranthene	0·876	0·917	0·927	0·951	—

Correlations with Indices of Mortality

	Cancer of lung (M.)	Bronchitis (M.)	Bronchitis (F.)	Pneumonia (M.)	Pneumonia (F.)
2nd order coefficients with one hydrocarbon constant					
Benzopyrene constant					
Smoke (total)	0·389	0·538	0·386	0·068	*Nil*
1: 12 Benzoperylene	0·055	*Nil*	0·017	0·223	,,
Pyrene	*Nil*	,,	*Nil*	0·081	,,
Fluoranthene	,,	,,	,,	*Nil*	0·240
Benzoperylene constant					
Smoke (total)	0·480	0·741	0·470	,,	0·002
3 : 4 Benzopyrene	0·394	0·511	0·259	0·073	0·237
Pyrene	0·002	0·040	*Nil*	0·058	*Nil*
Fluoranthene	0·065	0·077	,,	*Nil*	0·333
Pyrene constant					
Smoke (total)	0·674	0·748	0·671	0·262	0·173
3 : 4 Benzopyrene	0·654	0·625	0·624	0·306	0·443
1 : 12 Benzoperylene	0·542	0·397	0·535	0·363	0·288
Fluoranthene	0·318	0·225	*Nil*	0·026	0·581
Fluoranthene constant					
Smoke (total)	0·629	0·741	0·828	0·293	*Nil*
3 : 4 Benzopyrene	0·589	0·586	0·899	0·376	0·043
1 : 12 Benzoperylene	0·468	0·344	0·760	0·434	*Nil*
Pyrene	0·055	0·058	0·366	0·242	,,
Per cent reduction of smoke coefficients by eliminating one hydrocarbon					
Hydrocarbon constant					
3 : 4 Benzopyrene	56	38	49	90	100
1 : 12 Benzoperylene	46	15	37	100	100
Pyrene	25	14	11	61	64
Fluoranthene	30	15	0	56	100
Per cent reduction of other hydrocarbon correlations by eliminating 3 : 4 benzopyrene					
Benzopyrene constant					
1 : 12 Benzoperylene	93	100	97	69	100
Pyrene	100	100	100	88	100
Fluoranthene	100	100	100	100	59

fluoranthene, but in Table VI the results of eliminating the substances individually are different from those for lung cancer and bronchitis. When 1 : 12 benzoperylene is made constant all the other correlations disappear for males whilst fluoranthene remains predominant for females, and this suggests that these may be concerned with the incidence of some cases of pneumonia whereas benzopyrene is less important.

Trace elements and mortality in 23 areas

The amounts of 13 elements in the smoke samples were determined spectrographically at the Fuel Research Station of the Department of Scientific and Industrial Research by Mr. K. V. Aubrey. Filter papers were changed at intervals of a week or longer and the suspended matter collected throughout a whole year was aggregated for analysis. In 1956 when 10 stations were operating about 500 cubic feet of air per week passed through a standard 2-inch or a special 4-inch diameter filter according to the degree of air-pollution anticipated ; but in 1957 and 1958 when more stations were introduced about 3000 cubic feet per week were drawn through a 4-inch filter to obtain larger quantities of smoke for analysis. This was done at 23 of the 26 representative locations in Table I, and the results are given in Tables VII*a* and VII*b*. Analyses of copper and chromium are available for 14 of these.

TABLE VII*a*.—*Average Annual Amounts of Total Ash, Lead, Zinc, Copper, Titanium, Arsenic and Manganese per* 1000 *cubic metres of Air at* 23 *Localities in Northern England and Wales in* 1956–58.

		mg.		μg.				
Locality	Year	Total ash	Lead	Zinc	Copper	Titanium	Arsenic	Manganese
Conway Valley	1957	4·0	0·08	48	7	11	5	5
Llangefni	1956	6·0	0·27	76	—	10	11	10
Tattenhall	1956	8·0	0·22	130	—	30	23	27
Wetherby	1958	6·6	0·35	205	38	17	27	13
Ruthin	1957	8·5	0·47	73	14	33	13	10
Ripon	1958	6·5	0·40	100	21	15	23	13
Elland	1958	7·2	0·42	285	50	16	32	12
Flint	1956	12·5	0·29	230	—	43	33	40
York	1958	11·4	0·66	295	45	30	59	23
Chester	1956	12·0	0·50	250	—	30	46	39
Ormskirk	1957	10·0	0·52	225	45	25	35	16
Wrexham	1956	10·0	0·39	250	—	10	42	35
Lancaster	1958	8·9	0·63	185	33	23	39	12
Darwen	1958	11·6	0·71	185	32	36	37	52
Keighley	1958	13·0	0·71	140	44	42	41	43
Burnley	1958	16·1	0·95	185	34	71	44	32
Birkenhead	1956	13·0	0·60	240	—	20	68	35
Bootle	1956	22·0	0·58	490	—	50	130	50
St. Helens	1956	15·0	0·44	330	—	40	160	32
Warrington	1957	24·0	0·75	440	53	84	51	50
Leeds	1958	37·1	1·30	370	96	180	60	130
Liverpool (Princes Rd.)	1956	15·0	0·57	280	—	60	94	34
Salford (Regent Rd.)	1958	21·0	1·43	335	252	75	74	43

Note.—The localities are ranked in order of smoke density as in Table II.

TABLE VII*b*.—*Average Annual Amounts of Nickel, Antimony, Vanadium, Chromium, Molybdenum, Cobalt and Beryllium per* 1000 *cubic metres of Air at* 23 *Localities in Northern England and Wales in* 1956–58.

Locality	Year	μg. Nickel	Antimony	Vanadium	Chromium	Molybdenum	Cobalt	Beryllium
Conway Valley	1957	1·3	6·3	1·2	0·9	0·29	0·25	0·06
Llangefni	1956	2·3	4·0	3·4	—	0·20	0·70	0·20
Tattenhall	1956	4·1	5·3	6·9	—	0·40	1·30	0·20
Wetherby	1958	74·5	5·3	1·6	2·7	1·30	0·85	0·14
Ruthin	1957	1·8	3·3	1·2	1·6	0·60	0·74	0·15
Ripon	1958	31·2	4·5	1·5	2·9	1·19	0·83	0·14
Elland	1958	205·0	8·6	1·1	2·1	1·25	2·30	0·19
Flint	1956	6·9	7·0	5·9	—	1·00	1·20	0·50
York	1958	29·0	7·7	5·1	5·4	2·35	1·13	0·27
Chester	1956	12·0	12·0	7·6	—	1·90	1·60	0·60
Ormskirk	1957	16·0	12·5	4·6	2·7	1·40	0·97	0·26
Wrexham	1956	6·9	8·8	4·6	—	1·40	1·30	0·60
Lancaster	1958	17·3	7·2	5·4	2·3	1·56	0·78	0·21
Darwen	1958	11·2	9·5	8·8	4·0	3·40	1·05	0·31
Keighley	1958	32·5	13·0	3·7	4·1	2·50	2·05	0·31
Burnley	1958	13·5	8·6	6·8	4·8	4·45	1·55	0·40
Birkenhead	1956	10·0	12·0	31·0	—	2·00	1·60	0·60
Bootle	1956	23·0	20·0	42·0	—	1·80	2·00	1·00
St. Helens	1956	24·0	250·0	18·0	—	3·20	1·60	0·90
Warrington	1957	37·0	21·5	12·0	4·4	5·00	2·90	0·81
Leeds	1958	23·0	16·0	15·0	21·5	6·10	4·25	0·84
Liverpool (Princes Rd.)	1956	11·0	16·0	30·0	—	2·70	1·40	1·00
Salford (Regent Rd.)	1958	32·0	18·5	14·2	7·0	5·60	2·60	0·67

Note.—The localities are ranked in order of smoke density as in Table II.

For a few of the elements one or two places showed concentrations outside the normal range, as for example copper at Salford, titanium and manganese at Leeds, nickel at Elland, antimony at St. Helens, the presumed reason being proximity of special industries, but for the most part the distributions approximated to the normal type. The total ash ranged from 4 to 37 mg. per 1000 cubic metres of air; in Birkenhead, Liverpool, St. Helens and Salford it formed about 4 per cent of the total smoke, and in country districts about 7 to 10 per cent. The concentrations of 11 elements were correlated with the standardized mortality ratios in Table III with results shown in Tables VIII–X.

Correlations between trace elements and lung cancer

Table VIII shows that for each element except nickel and antimony concentration in air is correlated significantly with lung cancer mortality, the critical value at 1 in 20 level of significance being 0·40. Table IX reveals the effect on the total smoke coefficient (0·860) of making the amount of one element constant, and only for the first 5 elements, beryllium, arsenic, zinc, molybdenum and vanadium is this appreciable. The lower part of Table X shows that when the beryllium concentration is held constant the molybdenum correlation remains significant; and when molybdenum is constant the beryllium and vanadium

TABLE VIII.—*Correlations in 23 Localities of Northern England and Wales between Amounts of Trace Elements in Air, Standardised Mortality from Cancer, Bronchitis and Pneumonia in 1950–53 and Proportion of Males aged 15 and over in Social Class V in the Population.*

	1st order coefficients				
	Cancer of		Other cancer except stomach		
	Lung (M.)	Breast (F.)	(M.)	(F.)	Ash (total)
Beryllium	0·827	0·129	0·550	0·124	0·729
Arsenic	0·748	−0·080	0·321	0·086	0·534
Zinc	0·759	0·165	0·405	0·054	0·765
Molybdenum	0·684	0·028	0·520	0·109	0·746
Vanadium	0·770	0·053	0·556	−0·061	0·513
Cobalt	0·536	0·220	0·296	0·063	0·692
Nickel	−0·095	0·112	−0·241	−0·110	0·022
Manganese	0·521	0·351	0·400	0·162	0·928
Lead	0·626	−0·017	0·462	0·149	0·908
Titanium	0·527	0·082	0·338	0·106	0·936
Antimony	0·327	0·132	0·230	0·148	0·261
Social index	0·724	0·149	0·452	0·266	0·452

	Bronchitis		Pneumonia		
	(M.)	(F.)	(M.)	(F.)	Social index
Beryllium	0·767	0·642	0·760	0·783	0·670
Arsenic	0·743	0·513	0·684	0·643	0·745
Zinc	0·796	0·547	0·615	0·674	0·676
Molybdenum	0·842	0·816	0·482	0·447	0·399
Vanadium	0·620	0·463	0·805	0·711	0·763
Cobalt	0·710	0·599	0·498	0·497	0·215
Nickel	0·095	−0·015	−0·053	0·020	−0·232
Manganese	0·556	0·473	0·310	0·190	0·289
Lead	0·778	0·722	0·489	0·334	0·315
Titanium	0·602	0·597	0·495	0·512	0·223
Antimony	0·380	0·239	0·132	0·333	0·211
Social index	0·649	0·598	0·639	0·447	—

coefficients exceed 0·53 and the arsenic and zinc coefficients remain appreciable though not significant at the conventional level. Both beryllium and molybdenum appear to be associated with lung cancer incidence, and a suitably weighted combination of the two elements produces a high correlation of 0·720 with mortality after eliminating social index.

The connection with beryllium is supported by some experimental evidence of bronchogenic cancers in rats after inhaling beryllium dust for a long period, and several cases of lung cancer in men following berylliosis have been reported. Beryllium dust is known to be a respiratory irritant and many hundreds of cases of chronic berylliosis have occurred in processing the metal from ores (Hueper, 1957). It is of considerable interest to now find epidemiological indications of a connection between the small amounts of this element in ordinory air and the incidence of lung cancer amongst men who are continually inhaling it. Experimental evidence for any carcinogenic effects of molybdenum seems to be lacking, but this may be because it has not been sought for; the very high correlations noted below with bronchitis in both sexes are remarkable and the element might well be concerned also in lung cancer incidence.

Arsenic is correlated significantly (0·427) with lung cancer after eliminating the beryllium and molybdenum, and this tends to strengthen a view, long held, that this element in oxide form can have carcinogenic effects on the lung. Zinc gives a coefficient not quite significant at 1 in 20 level (0·379) and this might be meaningful in view of the evidence for a connection between its concentration in soil and the incidence of cancer of the stomach (Stocks and Davies, 1960). Vanadium also retains an appreciable correlation (0·347) after eliminating beryllium and molybdenum, and since it is a respiratory irritant some attention might be paid to it along with molybdenum as possible carcinogenic agents despite the present absence of clinical or experimental evidence.

Correlations between trace elements and other diseases

Table VIII shows no significant correlations between mortality from breast cancer and concentration of any trace element. The coefficient with manganese is reduced to 0·325 when social index is constant. Other cancer (excluding stomach) in females likewise shows no appreciable association with amount of smoke or of any element. In males however there is a smoke correlation of 0·603, which is reduced considerably by separate elimination of beryllium, molybdenum and vanadium. When social index and molybdenum concentration are

TABLE IX.—*Correlations between Mortality and Amount of Smoke in Air of 23 Localities when the Effect of one Trace Element on the Coefficient is Eliminated.*

	Lung cancer (M.)	Other cancer* (M.)	Bronchitis (M.)	Bronchitis (F.)	Pneumonia (M.)	Pneumonia (F.)
	1st order coefficients with smoke					
Smoke	0·860	0·603	0·919	0·843	0·712	0·564
Constant factor	2nd order coefficients with smoke, one element constant					
Beryllium	0·479	0·321	0·791	0·498	0·242	*Nil*
Arsenic	0·712	0·570	0·822	0·801	0·425	0·179
Zinc	0·733	0·489	0·847	0·768	0·504	0·189
Molybdenum	0·740	0·360	0·735	0·521	0·634	0·385
Vanadium	0·767	0·307	0·846	0·785	0·430	0·273
Cobalt	0·811	0·692	0·888	0·752	0·592	0·377
Nickel	0·880	0·602	0·936	0·848	0·711	0·568
Manganese	0·815	0·498	0·881	0·793	0·685	0·552
Lead	0·872	0·474	0·787	0·630	0·686	0·632
Titanium	0·812	0·535	0·871	0·753	0·609	0·366
Antimony	0·842	0·573	0·905	0·838	0·723	0·501
	Per cent reduction of smoke coefficients by eliminating one trace element					
Beryllium	44	47	14	41	66	100
Arsenic	17	9	10	5	40	68
Zinc	15	19	8	9	29	66
Molybdenum	14	40	8	38	11	32
Vanadium	11	51	8	7	40	55
Cobalt	6	0	3	11	17	33
Nickel	0	0	0	0	0	0
Manganese	5	21	4	6	4	2
Lead	0	17	14	25	4	0
Titanium	6	11	5	11	14	35
Antimony	2	5	1	0	0	11

* Excluding stomach.

held constant the correlations of other cancer with zinc, manganese and lead disappear but the coefficient with vanadium remains significant (0·396). This suggests that some forms of male cancer as well as lung may be affected by beryllium, molybdenum and vanadium.

Bronchitis shows in Table VIII correlations similar to those of lung cancer, and in the case of females the high coefficient with smoke is seen from Table IX to be reduced considerably by eliminating beryllium, molybdenum and lead. When social index and molybdenum are held constant the correlations with beryllium and lead virtually disappear, but molybdenum retains a high coefficient of 0·706 with female bronchitis after eliminating social index and beryllium. Bronchitis in males shows relations with the elements similar to those of lung cancer. With social index and beryllium constant the correlation with molybdenum is 0·712 ; when social index and molybdenum are eliminated arsenic and vanadium give coefficients of 0·563 and 0·469, beryllium gives 0·366 and zinc 0·360 ; and when social index and arsenic are constant molybdenum gives 0·850, beryllium 0·360 and zinc 0·425. It appears that molybdenum in the air is con-

TABLE X.—*Correlations in 23 Localities between Concentrations of Trace Elements in Air and Mortality when the Social Index and Other Trace Elements are Held Constant.*

2nd order inter-correlations between trace elements when social index is constant

	Molybdenum	Arsenic	Zinc	Vanadium	Cobalt	Manganese	Lead
Beryllium	0·495	0·756	0·652	0·610	0·552	—	0·311
Molybdenum	—	0·293	0·492	*Nil*	0·771	0·670	0·849
Both together	—	0·224	0·406	0·259	0·719	—	—
Vanadium	*Nil*	0·496	—	—	0·168	—	—
Zinc	—	0·541	—	—	—	—	—

3rd order coefficients with lung cancer, eliminating social index and one or more trace elements

Element constant	Beryllium	Molybdenum	Arsenic	Zinc	Vanadium	Cobalt	Lead
Beryllium	—	0·451	—	—	—	0·307	—
Molybdenum	0·552	—	0·389	0·337	0·538	—	—
Both together	—	—	0·427	0·379	0·347	0·096	—
Arsenic	0·587	0·591	—	—	—	—	—

Similar coefficients with other diseases

Bronchitis (males)

Beryllium	—	0·712	—	—	—	—	—
Molybdenum	0·366	—	0·563	0·360	0·469	0·296	0·294
Arsenic	0·360	0·850	—	0·425	—	—	—

Bronchitis (females)

Beryllium	—	0·706	—	—	—	—	—
Molybdenum	0·046	—	—	—	—	—	0·144

Pneumonia (males)

Beryllium	—	0·048	*Nil*	*Nil*	0·443	0·236	—
Vanadium	0·316	0·416	0·136	—	—	—	—

Pneumonia (females)

Beryllium	—	*Nil*	*Nil*	0·174	*Nil*	0·268	—

nected with bronchitis mortality in both sexes, and that arsenic, vanadium, beryllium and zinc may be concerned also in the case of males. The effects of beryllium on the respiratory tract have been referred to in reference to lung cancer. Vanadium pentoxide dust has for long been known to be an irritant to the upper respiratory tract, sometimes producing bronchitis or pneumonia amongst men working with it (Wyers, 1946; Sjoberg, 1950, 1956).

Pneumonia shows strongest primary correlations with beryllium, vanadium, arsenic and zinc, and the coefficients with smoke, which are not so great as for bronchitis and lung cancer, are reduced considerably by eliminating any of these elements. When social index and beryllium are held constant a significant coefficient remains only for vanadium (0·443) in the case of males, and all correlations disappear or become insignificant for females. It appears that beryllium is important for pneumonia in both sexes, and vanadium also in males.

Association of standardised mortality from cancer, bronchitis and pneumonia with amounts of smoke and atmospheric deposit in urban areas of England and Wales

In a previous paper (Stocks, 1959) correlations for cancer and bronchitis were given in respect of smoke collected by filters and undissolved deposit of larger particles in the county boroughs of England and Wales and districts of Lancashire and the West Riding of Yorkshire where data were available for one or more years from the reports of the D.S.I.R. The present study extends that analysis in the following ways: (1) records have become available for some new areas (2) cancer of the lung has been examined for each sex and cancer of the oesophagus distinguished where possible, (3) pneumonia has been added, (4) a similar study has been made of all the parts of Greater London for which data are available, (5) social conditions have been assessed by proportions of men in unskilled and partly skilled occupations. In towns where measurements had been recorded at multiple sites the mean value, after excluding instruments sited to investigate specific sources of pollution, was taken as the index of pollution for the town. The expected deaths for the standardised mortality ratios in 1950–53 were calculated by multiplying the England and Wales death rates at 12 sex-age groups in those years by the corresponding local populations in the 165 areas.

Atmospheric deposit (*undissolved*).—Table XI shows the correlations with the average amount of such deposit falling on unit area of the deposit gauge per month in 53 county boroughs. Coefficients exceeding 0·266 are significant at 1 in 20 probability level, those exceeding 0·345 at 1 in 100 level and those exceeding 0·437 at 1 in 1000 level. The coefficients with lung cancer, bronchitis and pneumonia after eliminating the effect of social factors are all highly significant. Cancer of the stomach is also related significantly with amount of deposit, but cancers of the oesophagus, intestine, breast and other sites show no appreciable correlation.

Table XII gives the coefficients in 74 districts of Lancashire and the West Riding of Yorkshire, and in the 71 of these which were urban in character. In the latter group coefficients exceeding 0·230 are significant at 1 in 20 level, those exceedings 0·300 at 1 in 100 level and those exceeding 0·376 at 1 in 1000 level. The partial coefficients when social index is held constant are highly significant for lung cancer and bronchitis, but with pneumonia they are small. Cancers of

organs other than the lung show no appreciable correlations when social index is constant, but stomach cancer in females gives a significant coefficient in the 74 districts when persons per acre are made constant.

TABLE XI.—*Correlations in 53 County Boroughs between Amounts of Atmospheric Deposit (Undissolved) and Standardised Mortality from Cancer, Bronchitis and Pneumonia in* 1950–53, *Persons per Acre and Proportion of Males Aged* 14 *and Over in Social Classes IV–V in* 1931.

		1st order coefficients			2nd order with deposit (constant idices)	
	Sex	Deposit	Persons per acre	Social index	P.P.A.	Social
Cancer of						
Lung (1950–54)	M. & F.	0·536	0·650	0·391	0·531	0·477
Oesophagus	M.	0·114	0·192	0·228	0·009	0·061
	F.	−0·193	−0·103	−0·133	−0·176	−0·171
Stomach	M.	0·414	0·077	0·360	0·408	0·344
	F.	0·383	0·111	0·401	0·374	0·301
Intestine	M. & F.	0·179	0·219	0·143	0·175	0·144
Breast	F.	−0·171	−0·075	−0·337	−0·158	−0·078
Other sites	M.	0·224	0·419	0·028	0·094	0·226
	F.	0·097	0·126	−0·025	0·072	0·109
Bronchitis	M.	0·621	0·346	0·504	0·579	0·570
	F.	0·540	0·245	0·379	0·511	0·483
Pneumonia	M.	0·503	—	0·464	—	0·431
	F.	0·685	—	0·487	—	0·647
Persons per ere	—	0·219	—	—	—	—
Social index	—	0·300	—	—	—	—

TABLE XII.—*Correlations in* 74 *Administrative Areas of Lancashire and the West Riding of Yorkshire between Amounts of Atmospheric Deposit (Undissolved), Standardised Mortality from Cancer, Bronchitis and Pneumonia in* 1950–53, *Persons per Acre and Proportion of Males Aged* 15 *and Over in Social Classes IV–V in* 1951.

		All areas with deposit data			Excluding Rural Districts		
		1st order, with		2nd order.	1st order		2nd order.
	Sex	Deposit	Persons per acre	Deposit (P.P.A. constant)	Deposit	Social index IV–V	Deposit (index constant)
Cancer of							
Lung (1950–54)	M. & F.	0·353	0·611	0·260	0·358	0·125	0·400
Stomach	M.	0·221	0·140	0·189	0·206	0·365	0·030
	F.	0·226	0·014	0·232	0·329	0·399	0·158
Breast	F.	0·041	−0·017	0·046	0·038	−0·261	—
Other sites	M.	0·258	0·313	0·187	0·237	0·310	0·102
	F.	0·114	0·099	0·099	0·068	0·254	−0·070
Bronchitis	M.	0·482	0·458	0·407	0·452	0·549	0·340
	F.	0·541	0·334	0·490	0·588	0·420	0·482
Pneumonia	M.	0·282	—	—	0·303	0·346	0·156
	F.	0·305	—	—	0·370	0·399	0·216
Persons per acre	—	0·305	—	—	—	—	—
Social index IV–V	—	—	—	—	0·498	—	—

Atmospheric particles large enough to fall into deposit gauges would seem unlikely to reach the lung, but abundance of them is usually accompanied by an excess of the finer particles which pass into smoke filters, and this may account partly for the high correlations with lung conditions. With stomach cancer there could be a direct agency, since such particles fall upon unprotected food and soil the hands, being then ingested.

TABLE XIII.—*Correlations in 30 County Boroughs between Amount of Smoke in Air, Standardised Mortality from Cancer, Bronchitis and Pneumonia in 1950–53, Persons per Acre and Proportion of Males Aged 14 and Over in Social Classes IV–V in the Population in 1951.*

		1st order coefficients			2nd order with smoke (constant indices)	
	Sex	Smoke	Persons per acre	Social index IV–V	P.P.A.	Class IV–V
Cancer of						
Lung (1950–54)	M.	0·494	—	0·448	—	0·326
	F.	0·491	—	0·468	—	0·309
	M. & F.	0·520	0·520	0·480	0·451	0·339
Oesophagus	M.	0·361	0·220	0·368	0·319	0·202
	F.	0·016	0·014	—	0·013	—
Stomach	M. & F.	0·685	—	0·539	—	0·546
Intestine	M. & F.	0·366	—	0·041	—	0·466
Other sites	M.	0·164	0·315	0·227	0·078	0·044
	F.	0·021	—	−0·160	—	0·042
Bronchitis	M.	0·604	0·313	0·587	0·564	0·405
	F.	0·576	0·380	0·499	0·525	0·410
Pneumonia	M.	0·493	0·409	0·563	0·426	0·240
	F.	0·332	0·153	0·539	0·303	0·036
Persons per acre	—	0·297	—	—	—	—
Social index IV–V	—	0·569	—	—	—	—

Smoke.—Table XIII shows the correlations with average annual amounts of smoke per unit volume of air in 30 county boroughs where filters had been operating. Coefficients exceeding 0·349 are significant at 1 in 20 level of probability, those exceeding 0·449 at 1 in 100 level and those exceeding 0·554 at 1 in 1000 level. The coefficient with lung cancer when population density is constant is 0·451 and when social index is constant it is 0·339. Bronchitis and cancers of the stomach and intestine also give significant correlations after eliminating the social factors, but the pneumonia figures are not so definite. Cancer of the oesophagus gives coefficients of doubtful significance and cancer of other sites shows no associations with smoke.

Table XIV gives coefficients in 45 districts of Lancashire and the West Riding where filters had been operating, and in 42 after excluding rural districts, those exceeding 0·287 being significant at 1 in 20 probability level and those exceeding 0·372 at 1 in 100 level. The correlations with lung cancer remain highly significant when social index is constant whichever criterion is used. Bronchitis and pneumonia coefficients are just below significance level. Cancers of the stomach, and of the breast and other sites in females show no appreciable associations with smoke, but other cancer in males gives a significant coefficient in the urban areas.

TABLE XIV.—*Correlations in 45 Administrative Areas of Lancashire and the West Riding of Yorkshire between Amounts of Smoke in Air, Standardised Mortality from Cancer, Bronchitis and Pneumonia in 1950–53, and Proportions of Males in Social Classes IV–V in 1951.*

	Sex	All areas with smoke data			Excluding Rural Districts		
		1st order		2nd order. Smoke (index constant)	1st order		2nd order. Smoke (index constant)
		Smoke	Class V index		Smoke	Class IV and V index	
Cancer of							
Lung (1950–54)	M. & F.	0·558	0·577	0·353	0·531	0·149	0·519
Stomach	M.	0·260	0·056	0·288	0·230	0·385	0·091
	F.	0·230	−0·017	0·310	0·225	0·483	0·041
Breast	F.	−0·002	−0·160	0·125	−0·006	−0·319	0·139
Other sites	M.	0·345	0·342	0·181	0·319	−0·026	0·390
	F.	0·034	−0·037	0·073	−0·001	0·068	−0·030
Bronchitis	M.	0·440	0·389	0·280	0·435	0·443	0·315
	F.	0·320	0·258	0·276	0·307	0·253	0·232
Pneumonia	M.	0·442	0·453	0·232	0·406	0·458	0·274
	F.	0·423	0·314	0·303	0·426	0·460	0·299
Social class IV–V	—	0·361	—	—	0·397	—	—
indices V	—	0·619	—	—	0·689	—	—

TABLE XV.—*Correlations in 40 Areas of Greater London between Amount of Smoke in Air, Standardised Mortality in 1950–53 from Cancer, Bronchitis, and Pneumonia, and Proportion of Males Aged 15 and Over in Social Classes IV and V in the Population.*

	Sex	1st order coefficients			2nd order with smoke (constant indices)	
			Social indices			
		Smoke	IV–V	V only	IV–V	V only
Cancer of						
Lung	M.	0·408	0·684	0·695	0·221	0·228
	F.	0·386	0·368	0·371	0·284	0·292
Stomach	M.	0·330	0·809	—	0·032	—
	F.	0·203	0·579	—	−0·025	—
All other sites	M.	0·164	0·514	—	−0·042	—
	F.	0·325	0·278	—	0·246	—
Bronchitis	M.	0·454	0·773	—	0·269	—
	F.	0·566	0·698	—	0·299	—
Pneumonia	M.	0·530	0·695	—	0·410	—
	F.	0·466	0·584	—	0·322	—
Social class IV–V	—	0·384	—	—	—	—
indices V	—	0·368	—	—	—	—

Table XV shows the correlations in 40 areas of Greater London where smoke data were available, the number comprising 15 districts in the Outer Ring, 24 Metropolitan Boroughs and a central area covering Holburn, City, Finsbury and Shoreditch. In view of the great daily movement of people, particularly of men, from one district to another it would hardly be surprising to find little or no correlation between mortality of residents and the average smoke density in the area of residence when social differences have been allowed for. Coefficients exceeding 0·304 are significant at 1 in 20 level, and the lung cancer and bronchitis

correlations are slightly below this level, though the pneumonia figures are significant for males and females. Stomach cancer shows no associations with smoke nor does cancer of other sites in males, but for other sites in females there is a small but not significant correlation. The Greater London results are in general accord with those from the other groups of areas, though as was expected the associations with smoke are weaker.

SUMMARY

Lung cancer mortality is strongly correlated with smoke density in the atmosphere in 26 areas of Northern England and Wales, in 45 districts of Lancashire and the West Riding of Yorkshire, and in 30 county boroughs, whilst similar though weaker correlations are found within Greater London. These relations are only partially explicable by social differences in the populations concerned. Bronchitis and pneumonia in males and bronchitis also in females show similar strong correlations with smoke. Cancers of the stomach and intestine are related significantly with smoke in the county boroughs, and cancers of sites other than lung and stomach in males are so related in the other groups of areas. In females cancers of the breast and other organs show no association with smoke. Pollution by larger particles is related significantly with lung and stomach cancers, bronchitis and pneumonia in 53 county boroughs and with lung cancer and bronchitis in 74 districts of Lancashire and the West Riding.

In the 26 localities the smoke samples were analysed in respect of polycyclic hydrocarbons and a statistical process of successive elimination was applied to discover which hydrocarbon was responsible for the smoke correlation with mortality rates. For lung cancer and bronchitis 3 : 4 benzopyrene emerges clearly as the substance of prime importance, with 1 : 12 benzoperylene contributing weakly for lung cancer, but for pneumonia 3 : 4 benzopyrene is apparently not important. The composite group of other cancers in males is correlated with several hydrocarbons, but cancers of the breast and other sites in females show no relations with any of them.

In 23 localities spectrographic analyses for 13 trace elements were made and a similar process of successive elimination was applied to those which showed appreciable correlations with mortality rates. For lung cancer beryllium and molybdenum emerge as the elements of most consequence, with arsenic, zinc and vanadium showing weaker associations. For bronchitis molybdenum appears to be the important element in both sexes whilst in males beryllium, arsenic, vanadium and zinc may also be concerned as for lung cancer. For pneumonia beryllium emerges as the important element in both sexes, with vanadium also concerned in males. With other cancer in males beryllium, molybdenum and vanadium show associations, but breast and other cancers in females show no relations with any element.

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SMOKING AND CANCER IN POLAND

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PART I.—STATISTICAL DATA ON SMOKING AND "TOBACCO TRACT" CANCER IN POLAND

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THE aim of this study is to discuss the results of the investigations on the connection between smoking and the appearance of some cancers. The investigations were carried out on patients of the Institute of Oncology in Gliwice which is the largest centre treating tumours in Poland.

In the first part of the study a short presentation is given of the consumption of tobacco in Poland and the appearance of "tobacco tract" cancer.

The view that smoking is a frequent cause of the appearance of lung cancer and some other tumours is ever increasing; it is not however accepted by all. That is why exhaustive investigations should be continued on the connection between smoking and the appearance of cancer.

If a distinct connection between smoking and for instance lung cancer is always found during the investigations in a variety of environments and in different countries then this fact cannot be considered as accidental. Such investigations should also make possible the recognition of the degree of the association between smoking and cancer morbidity (chances of morbidity depending upon the smoking habits) and explain what percentage of cases is in the given environment connected with smoking. If this percentage is greater in the environments with higher consumption of tobacco and lower in the environments in which act other carcinogenic factors—this will be the second serious argument for the carcinogenic action of tobacco smoke.

The third argument would be the finding of a connection between smoking and epidermoid cancer of the entire "tobacco tract", of the whole mucous membrane immediately in touch with tobacco smoke. It is difficult to imagine that such a connection could be accidental. Interesting results should be obtained by finding out the degree of this connection for cancer of various localizations and by comparison of the data obtained with the localization of tobacco tar in the respiratory tract of smokers (such studies have been started by Ermala and Holsti (1955).

If, as some claim, the connection between smoking and "tobacco tract" cancer is not a causative connection then it would have to be the effect of interdependence between smoking and some unknown factor causing the appearance of these tumours. The investigation of the connection between smoking and cancer should enable one to detect such a factor.

A. *Consumption of tobacco in Poland*

Some data concerning the consumption of tobacco in Poland are presented in Tables I and II. They show a marked increase in the consumption of tobacco (mostly cigarettes) after the second World War.

TABLE I.—*Tobacco Consumption in Poland (Polish and Imported Tobacco)—According to the Data Obtained from the Polish Tobacco Industry*

	1925	1929	1933	1937	1949	1953	1957
Cigars: in millions	52·2	78·0	36·5	28·9	24·9	22·5	31·2
Cigarettes (full smoking): in millions	7,754·3	10,190·1	3,712·4	3,747·8	19,881·2	30,088·4	41,539·0
Cigarettes (with mouthpieces): in millions			3,842·6	3,951·0	809·6	428·3	1,012·2
Tobacco: tons	11,242·2	13,011·3	10,927·0	12,583·2	973·0	342·5	..
Snuff: tons	389·7	445·6	314·0	252·8	92·6	95·4	..
Chewing tobacco: tons	..	..	8·0	..	4·8	7·5	..
Total consumption per head of the population (grammes of tobacco products)	603	692	549	562	849	1,216	1,491

Note: In order to make the table smaller the data are presented for every fourth year. For 1938–44 there are no data available; for 1945–47 the data are not accurate and for that reason are not presented here.

TABLE II.—*Tobacco Consumption in Poland in the Part Previously under Russian Rule (Doleżal, 1922)*

Year	Number of inhabitants	Tobacco consumption (pounds per head)
1829	4,137,634	0·72
1831	3,762,003	0·73
1832	3,914,665	0·94
1833	4,037,952	1·01
1834	4,059,517	0·83
1835	4,188,112	0·70
1845	4,798,658	0·79
1851	4,810,735	0·80
1855	4,647,454	0·93
1859	4,747,454	0·86

During the period between the first and the second World War mainly hand-made cigarettes were smoked (in cigarette tubes or rolled in tissue paper). Since the second World War hand-made cigarettes have been almost entirely given up for factory-made cigarettes. The smoking of pipes and cigars, disappearing at present, was rather popular during the period between the wars in the western part of Poland (Pomerania, Poznań District, Silesia, i.e. in the previous Prussian occupation[1]) but rarely met in the remaining parts of Poland.

Until recently there were no data on smoking habits of the individual smoker or on the structure of tobacco consumption in Poland. It is estimated that before the second World War the smokers constituted about 20 per cent of the entire population and that after the war the percentage increased mainly by a greater number of women smokers (personal communication from the Polish Tobacco Industry).

In 1957 preliminary investigations were carried out concerning the smoking habits and the structure of tobacco consumption in Poland (Staszewski and Wiśniewski, 1960). During these investigations the author collected data both on smoking habits (beginning, breaks, intensity and the manner of smoking)

[1] Up to 1918 the territory of Poland was devided for over 100 years by three occupants: Prussia, Russia and Austria.

as well as on such background data as professional activities, the places of residence (past and present) and the diseases the smokers had in the past.

Thus 2725 individuals of over 20 years of age were examined whose calling at the Institute of Oncology in Gliwice was most probably not connected with smoking as a causative agent: 1813 women (cancer and other diseases of the mammae—620, genital organs—600, skin—410, other organs which were not exposed to the direct action of the tobacco smoke—183) and 912 men (skin cancer—405, other skin diseases—117, cancer and other diseases of the genitals and mammary glands—82, lymphatic system—80, large intestine and rectum—71, skeletal system and soft tissues—53, other organs—104).

82·7 per cent of the examined men were smokers (present or past) who usually started smoking at about 20; the percentage of the smokers increased with age up to 60–70 years of life, the average intensity of smoking on the other hand decreased with age. Among women there were 8·4 per cent smokers. They had started smoking later and at various ages (average about 26 years old); the percentage of the smokers increased up to 35 years of age and then distinctly decreased. Differences were found in the consumption of tobacco between the different professional groups in both sexes. No distinct differences in smoking habits were noted between men living in the cities and in rural areas, but among women living in the rural areas there were markedly fewer smokers (1·8 per cent) than among those living in cities (12·8 per cent). Pipe and cigar smoking was met mainly among the population of Silesia (particularly among coal-miners and foundry workers and markedly fewer pipe smokers among office workers); the frequency of pipe smoking increased with age; it was not met before the thirty-fifth year of life, nor among women. 91·5 per cent of smokers who smoked cigarettes exclusively inhaled smoke as against only 34·7 per cent of those who smoked only pipe and/or cigars and 68·3 per cent of mixed smokers.

Some other data obtained will be presented by the Tables of the second part of this study.

B. "*Tobacco tract*" *cancer in Poland*

Up to 1950 the nomenclature of diseases and causes of death in Poland classified tumours only into 2 groups: "carcinoma and other malignant tumours", and "benign tumours or not diagnosed as malignant". Starting with 1951 there is a more exact classification taking into account, among others, the group of "carcinoma and other malignant tumours of the organs of respiration". Data on the mortality caused by the tumours of this group are shown in Table III. We can see a distinct increase both in the number of deaths due to cancer of the organs of respiration as well as an increase in their percentage in comparison with the total number of deaths or with the deaths due to all tumours. There is also a constant and distinct increase in the preponderance of men.

Since lung cancer, constituting the largest number of patients in this group, is curable so far in only a small percentage of the cases and the survival after the diagnosis amounts on the average to several months, it can be accepted that mortality in this case is only slightly smaller than morbidity.

Large differences in the mortality (and thus in morbidity) between the inhabitants of the capital or industrial Silesia (Katowice District) and agricultural districts (as Kielce District lying more or less half-way between Warsaw and Silesia) are most probably brought about first of all by the differences in the

TABLE III.—*Respiratory Cancer Mortality (No. V—99 Nomenclature of Diseases and Causes of Death), According to the Data Obtained from the Central Office of Statistics*

Year	Number of deaths due to cancer of the respiratory organs			Percentage of deaths due to respiratory cancer							
				In comparison with total mortality				In comparsion with mortality due to tumours			
	Total	Men	Women	Poland	City of Warsaw	Katowice District	Kielce District	Poland	City of Warsaw	Katowice District	Kielce District
1951	944	663	281	0·30	1·23	0·54	0·09	6·01	10·01	7·37	3·19
1952	1035	746	289	0·36	1·09	0·68	0·15	6·45	8·67	7·98	4·72
1953	1278	924	354	0·48	1·58	0·73	0·28	7·58	10·50	8·26	7·71
1954	1472	1113	359	0·53	1·75	0·80	0·20	8·19	12·17	8·93	5·19
1955	1577	1190	387	0·60	1·70	0·89	0·25	8·16	10·12	8·82	5·42
1956	1828	1418	410	0·73	2·23	1·24	0·32	8·96	12·34	10·87	6·32
1957	2041	1530	511	0·76	2·37	1·16	0·39	9·30	14·11	10·54	7·70

detection of tumours—difficult from the diagnostic point of view (as lung cancer). The data presented for the whole territory of Poland should therefore be considered as lower than in reality and the mortality and morbidity rates for Warsaw or Silesia are probably the closest to the actual rates for the whole country.

Since 1954 there exists in Poland a regulation requiring the notification of new cases of malignant tumours. It is not however strictly observed, e.g. in 1956 there were in Poland 20,409 deaths due to tumours (according to death statistics) but only 17,796 newly detected cases of malignant tumours were registered.

Lung cancer was, in 1956, the fourth in frequency of registered newly detected cases of malignant tumours among males (617 cases), being after cancer of the stomach (1444 cases), skin (822) and lip (747) and before cancer of the intestines (296 cases), larynx (270), oesophagus (220) and other organs.

The following are some data taken from the post-mortem statistics:

Nowicki (1931) claims that during the years 1896–1930 out of 30,957 post-mortem examinations performed at the Institute of Pathological Anatomy of the Jan Kazimierz University in Lwów, the percentage of lung cancer (93 cases) increased from 0·07 to 0·47. Out of a total of 2047 tumours the percentage of lung cancer increased from 1·8 to 5·9. Syrek (1931) mentions that during 1901–30 in the Department of Pathological Anatomy of the Jagiellonian University in Cracow, out of 28,455 post-mortem examinations 86 cases were found to be lung cancer (67 men and 19 women). The proportion of appearance of this tumour to the total number of post-mortem investigations increased during this period from 0·09 to 0·60 per cent, and in reference to the total number of cancer from 1·36 to 7·18 per cent.

Dąbrowska, Połtorzycka and Trojanowska (1932) presented data from 3 Warsaw Departments in which in the years 1911–31 out of 25,639 post-mortems performed in 212 cases lung cancer was found (169 men and 43 women). The frequency of lung cancer in respect to the total number of post-mortems increased from 0·5 per cent during the years 1911–13 to 1·4 per cent during 1929–31 and in respect to post-mortems due to malignant tumours from 5·7 to 12·8 per cent.

Thus one can see that the increase in the frequency of appearance of lung cancer is apparent also on the ground of post-mortem statistics.

Summary

The importance of epidemiological investigations into the problem of the carcinogenic action of tobacco smoking is presented.

Certain data are given in reference to tobacco consumption and smoking habits, concerning statistics on "tobacco tract" cancer mortality, and on post-mortem statistics of lung cancer in Poland.

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PART II.—TOBACCO SMOKING AND LUNG CANCER

Materials, Methods, Definitions

In more than 4 years between July 1954 and October 1958, 1031 men and 143 women were admitted to the Institute of Oncology in Gliwice with the diagnosis or suspicion of lung cancer.

I interviewed these patients as to the smoking habits, professions and places of residence in the same manner as the control group discussed in Part I of this study.

In 298 patients (275 men and 23 women) diagnosis of lung cancer was confirmed by histopathological findings. Seventy-one patients (including 15 men and 3 women with histopathologically proved lung cancer) were not interviewed because of their too short stay at the Institute. There remain then 281 patients (260 men and 21 women) interviewed with diagnosis confirmed histopathologically. These patients are the subject of the present study. Our material comprises over a half of the cases registered in Poland and possessing histopathological confirmation, e.g. in 1956 there were 127 such cases including 68 cases from our material.

The patients were divided into groups according to the histopathological diagnosis: 1. squamous-cell carcinoma, 2. adenocarcinoma, 3. microcellular carcinoma, 4. undifferentiated carcinoma or a type histologically not determined (foci carcinomatosi). The general characteristics of men belonging to these groups as well as to the control group are presented in Table I.

Table II presents the smoking habits of men suffering from lung cancer, and also the evaluation of statistical significance of the differences between them and the numbers characterizing smoking habits of the control group.

Those individuals who smoked for at least a year—and not less than an average of 1 g. of tobacco a day—were considered as "smokers". The individuals who besides cigarettes smoked pipe and/or cigars (each one smoked in a sufficient amount to consider the individual as a "smoker") were called "mixed smokers".

TABLE I.—*General Characteristics of Men Suffering from Lung Cancer and of the Control Group*

	Squamous cell carcinoma	Adeno-carcinoma	Micro-cellular carcinoma	Undifferentiated carcinoma or histological type not settled	Total carcinoma of bronchus	Control group
Number of individuals examined	137	20	32	71	260	912
Average age	56·5	52·8	50·7	55·6	55·3	53·4
Number and % of inhabitants of towns	85 (62·0)	12 (60·0)	24 (75·0)	46 (64·8)	167 (64·2)	568 (62·3)
Number and % of inhabitants of Upper Silesia	104 (75·9)	15 (75·0)	24 (75·0)	49 (69·0)	192 (73·8)	641 (70·3)
Number and % of office workers	29 (21·2)	3 (15·0)	7 (21·9)	16 (22·5)	55 (21·2)	111 (12·2)
Number and % of farmers .	7 (5·1)	4 (20·0)	4 (12·5)	7 (9·9)	22 (8·5)	126 (13·8)

TABLE II.—*Smoking Habits of Men Suffering from Lung Cancer and of the Control Group*

	Squamous cell carcinoma	Adeno-carcinoma§	Micro-cellular carcinoma§	Undifferentiated carcinoma or histclogical type not settled	Total carcinoma of bronchus	Control group
Number of individuals examined	137	20	32	71	260	912
Number and % of smokers .	137 (100·0)‡	20 (100·0)	29 (90·6)	69 (97·2)‡	255 (98·1)*‡	754 (82·7)
Average intensity of smoking .	17·2‡	16·0	15·0	15·9†	16·5*‡	12·2
Average index of smoking .	666·2‡	510·5	493·4	587·6†	612·0‡	406·3
Number and % of heavy smokers (with the index over 300)	133 (97·1)‡	17 (85·0)	24 (82·8)	61 (88·4)‡	235 (92·2)‡	447 (59·3)
Number and % of smokers smoking only cigarettes .	117 (85·4)†	15 (75·0)	23 (79·3)	63 (91·3)†	218 (85·5)†	552 (73·2)
Number and % of smokers smoking only pipe and/or cigars	2 (1·45)‡	0 (0·0)	2 (6·9)	0 (0·0)	4 (1·6)‡	101 (13·4)
Number and % of smokers inhaling smoke	133 (97·1)‡	18 (90·0)	28 (96·6)	66 (95·6) †	245 (96·1)‡	609 (80·8)
Average duration of smoking habits§	38·7	32·9	31·0	38·1	37·1	32·2

* The calculations of significance have been taken from the study by Dr. Różanowicz (unpublished).

† The difference with the control group is significant—exceeding over three times the standard error of difference calculated in the equation :

$$SE = \sqrt{\frac{p_1 \cdot q_1}{n_1} + \frac{p_2 \cdot q_2}{n_2}}$$

‡ The difference with the control group is exceedingly significant, exceeding at least six times the standard error of difference.

§ For adenocarcinoma, for microcellular cancer, and for average duration of smoking habits, significance of differences was not computed.

The duration of smoking was determined after subtracting the breaks in smoking lasting longer than a year.

The intensity of smoking or the amount of grammes of tobacco smoked daily is given as an average for the whole period of smoking. It was accepted that 1 cigarette = 1 g. of tobacco, and 1 cigar = 4 g. of tobacco.

The most frequently met classification of the individuals in reference to smoking habits is the one according to the intensity of smoking (e.g. smoking 1 package of cigarettes, or smoking more than 1 package a day, etc.). This does not however take into account a very important factor of time, and causes placing together into one group smokers who all smoke more than 1 package of cigarettes a day indeed—but one of them smokes for 5 years while another one for 40 years. In order to avoid this and for the purpose of better characterization of smoking by one number—giving some idea as to the intensity and the duration of the smoking habit as well—the term " smoking index " was introduced (Staszewski, 1958). This index is the product of intensity of smoking multiplied by the duration of smoking. If the individual smoked, for example, 12 cigarettes a day on the average for 40 years then the smoking index would be $12 \times 40 = 480$. This index multiplied by 0·365 gives the amount of tobacco in kilograms smoked during the life of the individual.

Those who presented the smoking index over 300 were considered as " heavy smokers ".

Individuals living steadily in a town or usually in a town and in the country not longer than 10 years, are considered as " town inhabitants ".

Individuals who lived steadily in Katowice or Opole Districts or not longer than 10 years outside of these Districts are considered as " inhabitants of Upper Silesia ".

For the classification of the individuals according to occupations, the occupation performed the longest was taken into consideration.

Results

A. *Men* (Table II)

The percentage of smokers was considerably higher among the individuals afflicted by lung cancer than in the control group. It is striking that there is not one non-smoker among patients with squamous-cell carcinoma, who constitute over 50 per cent of patients with lung cancer.

The average smoking index among smokers was 50 per cent higher in patients with lung cancer than in the control group.

The percentage of " heavy " smokers (i.e. with the smoking index over 300) was also about 50 per cent higher in patients with lung cancer.

Average intensity of smoking among patients with lung cancer was about one-third higher than in the control group.

The average duration of smoking depends on three factors: Average age of the persons examined (among patients with lung cancer it was almost 2 years higher than in the control group), breaks in smoking, and the age of beginning smoking.

Individuals with lung cancer began smoking on the average 1·8 years earlier than those in the control group.

TABLE III.—*Smoking Habits of Women Suffering from Lung Cancer and of the Control Group*

	Squamous cell carcinoma	Adeno-carcinoma	Micro-cellular carcinoma	Undifferentiated carcinoma or histological type not settled	Total carcinoma of bronchus	Control group
Number of individuals examined	1	11	5	4	21	1813
Number and % of smokers	1 (100·0)	1 (9·1)	1 (20·0)	3 (75·0)	6 (28·6)	153 (8·4)
Average index of smoking (calculated for smokers)	540	30	390	371·7	345·8	142·6
Average index of smoking (calculated for smokers and non-smokers together)	540	2·7	78·0	278·8	98·8	12·0
Number and % of heavy smokers (with the index over 300)	1 (100·0)	0 (0·0)	1 (100·0)	2 (66·7)	4 (66·7)	20 (13·2)
% of individuals examined (smokers and non-smokers together) being heavy smokers	100·0	0·0	20·0	50·0	19·0	1·2
Average intensity of smoking	15	3	10	15·7	12·5	8·5

Among 137 patients with squamous-cell carcinoma only 2 smoked less than 25 years: one—14 years (a year after pneumonectomy performed in the thirty-third year of life carcinoma clarocellulare renis was found); the second—21 years. Among the patients with adenocarcinoma 5 patients had smoked for less than 25 years, among the patients with microcellular carcinoma—3, with undifferentiated—4.

The manner of smoking.—There were markedly fewer pipe smokers and/or cigar smokers among the patients with lung cancer than in the control group.

Inhaling of smoke as well as smoking of cigarettes was much more frequent among patients with lung cancer than in the control group.

The statistical significance of the differences in the consumption of tobacco between the patients having lung cancer and the control group are given in Table II.

Histological subgroups.—Patients with squamous-cell carcinoma, who comprised over half of the patients discussed, present the highest consumption of tobacco. All without exception smoked tobacco and almost all of them were " heavy " smokers (i.e. smoking index over 300), smoked cigarettes, and inhaled smoke.

Similar, yet a little lower, numbers characterize the second subgroup—"undifferentiated carcinoma or histological type not determined".

Two remaining subgroups are small and it is difficult to draw conclusions in this case. It seems that they smoke less than the patients with squamous-cell carcinoma and among them pipe smokers and/or cigar smokers are met more frequently. On the other hand they smoke more than the examined individuals in the control group.

Other data.—17·6 per cent of men with lung cancer had had pneumonia as had 13·1 per cent in the control group (the difference statistically is of no significance). Other diseases of lungs were rarely mentioned by the individuals in both groups.

Among the patients suffering from the lung cancer the division of blood groups was the following (for comparison the division of blood groups in Poland are given in brackets (Sabliński, 1959): A—41·7 per cent (37·1 per cent), B—19·7 per cent (18·5 per cent), AB—6·3 per cent (7·6 per cent), O—32·3 per cent (36·7 per cent). Thus no connection was found between lung cancer and some blood groups.

B. *Women* (Table III)

The small number of cases makes detailed analysis impossible and only allows a few general conclusions to be reached.

The numerical proportion of men to women for the total number of patients suffering from lung cancer amounted to 12·4 : 1, for adenocarcinoma only 1·8 : 1 and for the total of the remaining histological types 24·0 : 1.

The percentage of smokers, average index, and average intensity of smoking were higher among women suffering from lung cancer than in the control group.

There is almost no difference in women patients with adenocarcinoma in reference to smoking and the control group, but in all the remaining histological subgroups the consumption of tobacco is higher. The only woman with squamous-cell carcinoma smoked and had the index of smoking above 300 (such index was met in 1·2 per cent of women in the control group).

Discussion

A. *Connection between smoking and lung cancer*

Patients of both sexes suffering from lung cancer smoke markedly more than the " general population " represented by the control group. Is it possible, however, to compare these two groups? Do they represent the same population?

As can be seen from Table I, the differences in the social background of the two groups are not great. Average age, the percentage of the inhabitants of towns and the percentage of the inhabitants of Upper Silesia are very similar. The differences in the professional structure are greater—but they could not serve for the explanation of the differences noted in tobacco consumption. In none of the subgroups of professions such a high index or percentage of smokers was found as among individuals suffering from lung cancer (average index of smoking varied from 364·4 among metal workers to 512·9 among railroad workers, and the percentage of smokers from 70·3 among the office workers up to 89·1 among the colliers—thus in the control group in not one of the subgroups of professions were the values as great as among the lung cancer patients).

It may be then accepted that, as ascertained in our material, accurate statistically highly significant connection between tobacco smoking and lung cancer is not an accidental occurrence. Whether it is a causal connection—statistical and epidemiological studies cannot give a decisive answer. It can only be stated that our studies are in conformity with the view that smoking plays a most important role in the genesis of lung cancer.

The connection between smoking and lung cancer is quite distinct for patients of both sexes with squamous-cell carcinoma and with cancer included in the subgroup as "undifferentiated carcinoma and the type histologically not determined "; it is, however, less distinct for microcellular carcinoma and adeno-

carcinoma. In reference to the latter, conclusions cannot be drawn upon ascertaining larger consumption of tobacco among men—due to the small number of cases and due to the low tobacco consumption among women with adenocarcinoma.

Our material is not sufficient to determine whether lung cancer possesses any connection with pipe and/or cigar smoking; if there were such a connection it would be markedly smaller than in reference to cigarette smoking.

B. *The chances of morbidity and the percentage of cases connected with smoking*

Relative chances of morbidity in reference to lung cancer which the smoker had in comparison with the non-smokers may be calculated for our material by employing a formula given by Cornfield (1951):

$$A = \frac{p_1}{p_2} \cdot \frac{(1 - p_2)}{(1 - p_1)}$$

where A = relative amount by which the prevalence of lung cancer is augmented by the attribute of smoking.

p_1 = proportion of smokers among individuals examined with lung cancer.

p_2 = proportion of smokers among individuals examined in the control group.

Using this formula we find that a smoker possessed about 10-fold greater chances of becoming afflicted by lung cancer than a non-smoker.

The proportion of cases connected with smoking will then be:

$$y = \frac{A - 1}{A} \times p_1 = 88{\cdot}2 \text{ per cent.}$$

And thus in almost 90 per cent of male individuals we found a connection between smoking and lung cancer.

The same problem may be discussed in another way. Let us calculate how many men with lung cancer we would expect to meet if smoking among the entire male population were the same as among women and if there were a close correlation between the appearance of lung cancer (except adenocarcinoma) and high tobacco consumption represented by a higher index than 300. Such an index appeared in 49·1 per cent of the total number of examined men and in 1·2 per cent of the total number of women in the control group—proportion 41 : 1. And therefore a decrease in the frequency of appearance of a higher than 300 index by 41 times would also bring about a decrease in the number of men afflicted by lung cancer. We would then have not 240 but only 6 men suffering from lung cancer of a type other than adenocarcinoma and therefore a similar number as in women (10 cases after subtracting cases of adenocarcinoma). Total elimination of smoking as a carcinogenic factor would also decrease a little the number of 26 cases of lung cancer in men and 21 cases in women. It would result then that smoking played (with the above foundation) the main role in over 90 per cent of cases of lung cancer among men ($>234/260$) and in over 80 per cent of the total number of cases ($>234/281$). The numbers obtained by both methods are similar.

Conclusions

Our observations prove that in Poland there is also a distinct connection between cigarette smoking and the appearance of lung cancer. The connection is observed in men as well as in women—most distinctly for squamous-cell carcinoma. Contrary to the findings of other authors the connection of smoking with microcellular carcinoma has been less distinct in Poland than with adenocarcinoma; both these subgroups were, however, too small to draw far-reaching conclusions.

The markedly more frequent appearance of lung cancer among men can be easily explained when accepting that smoking is frequently the cause of cases presenting this neoplasm.

The period from the moment of starting smoking to the appearance of symptoms of lung cancer was usually longer than 25 years (on the average it amounted to 37·1 years). Therefore a conclusion can be reached that in studying dependence between smoking habits and morbidity due to lung cancer in the examined population one should take into consideration consumption of tobacco during the last 30–40 years and not the present consumption.

It is evaluated that men smokers possessed about 10-fold greater chances of becoming afflicted by lung cancer than the non-smokers and that smoking has been connected with about 80 per cent of the total number of lung cancer cases.

Summary

Results of a retrospective study conducted in Poland on tobacco smoking and lung cancer are presented. This study shows a very distinct correlation between cigarette smoking and lung cancer.

It is considered that men smokers possessed about 10-fold greater chances of becoming afflicted with lung cancer than non-smokers and that smoking was connected with about 80 per cent of the total number of lung cancer cases.

The long period from the moment of starting smoking to the appearance of symptoms of cancer points to the necessity of taking into consideration tobacco consumption during the last 30–40 years during the study of correlation between smoking habits and morbidity due to lung cancer.

The index of smoking (intensity × duration) seems to be a better criterion of classification of smokers than intensity of smoking.

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PART III.—CANCER OF THE "TOBACCO TRACT" (EXCLUDING LUNG CANCER) IN MEN

During the years 1957–58 data have been collected concerning tobacco consumption, occupation, and places of residence of 394 men suffering from carcinoma and pre-cancerous conditions of the lip, 83 men with carcinoma and pre-cancerous

conditions of the oral cavity, 19 with carcinoma of the tonsils, and 207 with carcinoma of the larynx.

A group of 912 men over 20 years old examined in the first half of 1957, whose reason for calling at the Institute of Oncology was most probably not connected with smoking, serves as a control group (group " C "). This group is described in general outlines in the first part of this study which discusses tobacco consumption in Poland.

Additional comparison group (group " D ") for patients with carcinoma of lip will be represented by 200 farmers afflicted by carcinoma of skin.

The manner of collecting data both for the group of examined individuals as well as in both control groups and the definitions " smokers ", " heavy smokers ", " index of smoking ", " the inhabitants of towns ", etc. are as described in Part II of this study on lung cancer.

Results and Conclusions

In Table I some general data are presented in reference to patients afflicted by cancer of various sites, and in Tables II and III data on their smoking habits. We shall discuss cancer of each site in turn.

TABLE I.—*General Characteristics of Men with Cancer of the " Tobacco Tract " and of the Control Group*

	Carcinoma and precancerous conditions of lip	Oral cavity			Carcinoma of the tonsils	Carcinoma of larynx	Control group
		Carcinoma	Pre-cancerous conditions	Total			
Number of individuals examined	394	58	25	83	19	207	912
Average age . .	55·8	57·9	53·0	56·4	62·5	56·4	53·4
Number and % of inhabitants of towns	114 (28·9)	38 (65·5)	15 (60·0)	53 (63·9)	15 (78·9)	138 (66·7)	568 (62·3)
Number and % of inhabitants of Upper Silesia	217 (55·1)	42 (72·4)	17 (68·0)	59 (71·1)	13 (68·4)	149 (72·0)	641 (70·3)
Number and % of office workers	16 (4·1)	11 (19·0)	5 (20·0)	16 (19·3)	6 (31·6)	26 (12·6)	111 (12·2)
Number and % of farmers	126 (32·0)	8 (13·8)	2 (8·0)	10 (12·0)	1 (5·3)	23 (11·1)	126 (13·8)

1. *Cancer of lip*

In 387 patients changes concerned the lower lip and in 7 the upper lip. Histopathological diagnosis of squamous-cell carcinoma was positive in 306 patients, uncertain in 41 patients (probably carcinoma, suspicions of carcinoma), and in 47 patients only precancerous conditions were found (hyperkeratosis et proliferatio epithelii, paratypia epithelii, papilloma, leukoplakia). The patients in question were divided into 2 subgroups: farmers and non-farmers—since cancer of lip is particularly frequent in farmers, which suggests the pertinence of analysing them separately.

The data concerning the tobacco consumption in each of these subgroups is presented in Table II. Before discussing it, however, a few words will be devoted to the influence of atmospheric factors on the appearance of cancer of lip.

TABLE II.—*Smoking Habits of Men Suffering from Carcinoma and Precancerous Conditions of Lip and of the Control Group*

	Control group "C"	Carcinoma and precancerous conditions of lip. Total	Non-farmers				Farmers				Additional group for comparison "D"
			Carcinoma*	Carcinoma*	Precancerous conditions*	Total	Carcinoma *	Carcinoma ?*	Precancerous conditions*	Total	
Number of individuals examined	912	394	207	27	34	268	99	14	13	126	200
Number and % of smokers .	754	368‡	197	25	32	254§	88	14	12	114†	151***
	(82·7)	(93·4)	(95·3)	(92·6)	(94·1)	(94·8)	(88·9)	(100·0)	(92·3)	(90·5)	(75·5)
Average intensity of smoking	12·2	14·1‡	14·8	13·4	14·6	14·6‡	12·7	14·3	12·3	12·9	10·1***
Average index of smoking .	406·3	513·8‡	532·4	461·2	551·4	526·5‡	480·2	543·9	445·8	484·1‡	423·3**
Number and % of heavy smokers (with the index over 300)	447	274‡	155	14	24	193‡	64	10	7	81†	90**
	(59·3)	(74·5)	(78·7)	(56·0)	(75·0)	(76·0)	(72·7)	(71·4)	(58·3)	(71·1)	(59·6)
Number and % of smokers smoking only cigarettes	552	288	147	18	24	189	78	12	9	99‡	109**
	(73·2)	(78·3)	(74·6)	(72·0)	(75·0)	(74·4)	(88·6)	(85·7)	(75·0)	(86·8)	(72·2)
Number and % of smokers smoking only pipe and/or cigars	101	47	28	4	5	37	6	2	2	10	22
	(13·4)	(12·8)	(14·2)	(16·0)	(15·6)	(14·6)	(6·8)	(14·3)	(16·7)	(8·8)	(14·6)
Number and % of smokers inhaling smoke	609	300	159	22	26	207	74	9	10	93	107
	(80·8)	(81·5)	(80·7)	(88·0)	(81·3)	(81·5)	(84·1)	(64·3)	(83·3)	(81·6)	(70·9)

Note : The calculations of significance marked †, ‡, §, have been taken from the study by Dr. Różanowicz (personal communication).

* For this column significance of difference was not computed.

† The difference with the control group "C" is significant—exceeding two to three times the standard error of difference calculated in the equation

$$\text{S.E.} = \sqrt{\frac{p_1 q_1}{n_1} + \frac{p_2 q_2}{n_2}}$$

‡ The difference with the control group "C" is distinctly significant—exceeding three to six times the standard error of difference.

§ The difference with the control group "C" is exceedingly significant—exceeding at least six times the standard error of difference.

, *, Definitions of significance as above—for differences between the 126 farmers with carcinoma and precancerous conditions of lip and the additional group for comparison "D". ** Means that the difference is significant, and *** that it is distinctly significant.

In the group of non-farmers the percentage of individuals exposed because of their occupation to the action of atmospheric factors was markedly higher than in the control group " C ". There were in this group for instance more workers employed in the building industry (16·4 per cent in comparison with 9·0 per cent in the control group after subtracting the number of farmers), and less office workers (5·9 per cent in comparison with 14·1 per cent in the control group) or inhabitants of towns. This type of connection with the exposure to atmospheric factors was not found in the other cancers discussed later on. It is worth mentioning that among the patients with cancer and precancerous conditions of lip, farmers constitute almost one-third of the total number of patients (126 out of 394), while in the control group only about one-seventh (126 out of 912). The farmers are really particularly exposed to the action of atmospheric factors. It can be supposed then that these factors (probably the ultraviolet rays of the sun spectrum) play a large role in the appearance of precancerous conditions and cancer of lip.

Let us now take into account the tobacco consumption among the patients mentioned above.

The percentage of smokers, average intensity of smoking, average index of smoking and the percentage of heavy smokers (i.e. with the index of smoking above 300) were markedly higher among patients with cancer and precancerous conditions of lip than in the control group " C ". This is observed in all the subgroups of Table II (only in two small subgroups the percentage of heavy smokers was a bit lower than in the control group). The differences compared with the control group were larger for non-farmers (statistically significant) than for farmers for whom these differences were statistically of no importance or on the boundary of significance.

Let us compare now the farmers suffering from cancer and precancerous conditions of lip not with the control group " C " but with the additional group for comparison (" D "): 200 farmers with cancer of skin, not a large group really, but more corresponding in reference to the mode of life, conditions of work, etc., and thus representing better the consumption of tobacco among the farmers. Such a comparison brings into prominence higher tobacco consumption among farmers with cancer and precancerous conditions of lip—e.g. the percentage of smokers among them is distinctly higher than in the additional group (" D ") for comparison (the difference here exceeds 3·7 times the error of difference).

The manner of smoking.—Differences in reference to percentage of smokers using only cigarettes, percentage of smokers using only pipe and/or cigars, and the percentage of smokers inhaling smoke were statistically not significant; however among patients with cancer and precancerous conditions of lip there were a few more cigarette smokers and smokers inhaling smoke than in the control group. This is contrary to the results obtained by other investigators who have stated that the connection between cancer of lip and smoking is particularly distinct in smokers using a pipe and/or cigars. The reason for this disagreement is not clear. It may be caused for instance by a small number of pipe and cigar smokers among our patients—studies carried on larger material could perhaps explain that.

Chances of morbidity and the percentage of cases connected with smoking will be calculated similarly as for lung cancer in Part II of this study.

Relative chances of cancer and precancerous conditions of lip which a smoker has in comparison with a non-smoker amount to, after Cornfield's (1951) formula,

$$A = \frac{0{\cdot}934}{0{\cdot}827} \cdot \frac{(1 - 0{\cdot}827)}{(1 - 0{\cdot}934)} = 2{\cdot}96.$$

The percentage of cases connected with smoking amounted to:

$$y = 61{\cdot}8 \text{ per cent.}$$

Analogic computations for the subgroups of farmers when compared with the additional comparison group " D " give:

$$A = \frac{0{\cdot}905}{0{\cdot}755} \cdot \frac{(1 - 0{\cdot}755)}{(1 - 0{\cdot}905)} = 3{\cdot}09$$

$$y = 61{\cdot}2 \text{ per cent.}$$

Concluding, among our patients we find a distinct connection between the appearance of carcinoma and precancerous conditions of lip, and tobacco smoking and exposure to the action of atmospheric factors. Pipe and/or cigar smoking was not found to be more connected with the discussed diseases than smoking of cigarettes. The smoker had 3 times greater chance of becoming afflicted than the non-smoker. About 60 per cent of cases were connected with smoking.

2. *Cancer of the oral cavity*

In 58 patients the diagnosis of squamous-cell carcinoma was proved by histopathological examination and in 25 patients there were found only precancerous changes (leukoplakia, suspicious epithelial proliferation, papilloma). Differences in the smoking habits of both of these small subgroups were not great (Table III).

The percentage of smokers, average intensity of smoking, average index of smoking and the percentage of heavy smokers were significantly higher among patients suffering from cancer and precancerous conditions of the oral cavity than in the control group. But the differences in the manner of smoking were not significant from the statistical point of view. The percentage of those smoking only pipe and/or cigars and not inhaling smoke was however a little higher among the discussed patients. This is in agreement with the statement by other investigators that there is a more distinct connection between cancer of the oral cavity and smoking pipe and/or cigars than with cigarette smoking.

The relative chances of a smoker to become afflicted by cancer and precancerous conditions of the oral cavity amounted to:

$$A = \frac{0{\cdot}988}{0{\cdot}827} \cdot \frac{(1 - 0{\cdot}827)}{(1 - 0{\cdot}988)} = 17{\cdot}2.$$

This number may be considered as only a near estimate because with the small number of non-smokers it is very dependent on chance (a slight change in the percentage of smokers among patients markedly changes the number in the denominator). The percentage of cases connected with smoking amounted to:

$$y = 93{\cdot}1 \text{ per cent.}$$

Concluding, among the discussed patients there was a distinct connection between smoking, and cancer and precancerous conditions of the oral cavity.

TABLE III.—*Smoking Habits of Men with Cancer of the Oral Cavity, Tonsils and Larynx and of the Control Group*

	Oral cavity					
	Carcinoma*	Precancerous conditions*	Total	Carcinoma of the tonsils*	Carcinoma of the larynx	Control group
Number of individuals examined	58	25	83	19	207	912
Number and % of smokers	57 (98·3)	25 (100·0)	82 (98·8)§	19 (100·0)	206 (99·5)§	754 (82·7)
Average intensity of smoking	15·1	14·9	15·0‡	16·4	15·7§	12·2
Average index of smoking (calculated for smokers)	544·7	492·4	535·4‡	600·8	585·3§	406·3
Number and % of heavy smokers (with the index over 300)	47 (82·5)	20 (80·0)	67 (81·7)‡	13 (68·4)	183§ (88·8)	447 (59·3)
Number and % of smokers smoking only cigarettes	39 (68·4)	22 (88·0)	61 (74·4)	13 (68·4)	182‡ (88·3)	552 (73·2)
Number and % of smokers smoking only pipe and/or cigars	12 (21·1)	2 (8·0)	14 (17·1)	3 (15·8)	4* (1·9)	101 (13·4)
Number and % of smokers inhaling smoke	40 (70·2)	22 (88·0)	62 (75·6)	15 (78·9)	199§ (96·6)	609 (80·8)

Note : The calculations of significance have been taken from the study by Dr. Różanowicz (personal communication).

* For this column significance of difference was not computed.

† The difference with the control group is significant—exceeding two to three times the standard error of difference calculated in the equation

$$\text{S.E.} = \sqrt{\frac{p_1 q_1}{n_1} + \frac{p_2 q_2}{n_2}}$$

‡ The difference with the control group is distinctly significant—exceeding three to six times the standard error of difference.

§ The difference with the control group is exceedingly significant—exceeding at least six times the standard error of difference.

The smoker had about 17 times greater chance of becoming afflicted than the non-smoker. About 90 per cent of cases were connected with smoking.

3. *Carcinoma of tonsils*

In this small group in all cases the diagnosis of squamous-cell carcinoma was confirmed by histopathological findings.

All the patients were smokers. Average intensity and average index of smoking and the percentage of heavy smokers were markedly higher than in the control group, but the manner of smoking did not differ in both groups. Due to the small number of patients with carcinoma of tonsils statistical appraisal of these correlations is not possible.

4. *Carcinoma of larynx*

In all the patients the diagnosis of squamous-cell carcinoma was confirmed by histopathological findings. Extrinsic larynx cancer was also included here. There were only slight differences in the smoking habits of the patients with cancer of the larynx of various localizations.

The percentage of smokers, average intensity of smoking, average index of smoking and percentage of heavy smokers were markedly higher among these patients than in the control group (the differences being statistically extremely significant).

The percentage of smokers using only cigarettes as well as the percentage of those inhaling smoke was among the patients with cancer of larynx markedly higher than in the control group; these percentages were also higher than in patients with cancer of lip, cancer of the oral cavity, or tonsils—and very similar to the percentages observed in lung cancer (see Part II of this study).

The relative chances of morbidity for smokers amounted to:

$$A = \frac{0{\cdot}995}{0{\cdot}827} \cdot \frac{(1 - 0{\cdot}827)}{(1 - 0{\cdot}995)} = 41{\cdot}6.$$

The number obtained above is only of orientation significance because of the very small number of non-smokers—it would greatly change if for instance instead of one there were two non-smokers. The obtained number however proves that the smoker had many more chances of becoming afflicted by cancer of larynx than the non-smoker.

The percentage of cases connected with smoking amounted to:

$$y = 97{\cdot}1 \text{ per cent.}$$

Concluding, among the discussed patients cancer of larynx is very distinctly connected with smoking and particularly with cigarette smoking and inhalation of smoke. The smoker had about 40 times greater chance of becoming afflicted by cancer of larynx than the non-smoker. Over 90 per cent of cases afflicted by the said tumour were connected with smoking.

5. *Women*

Among 31 examined women, not included in the Tables (13 with carcinoma and precancerous conditions of lip, 3 with carcinoma of the oral cavity, 2 of the tonsils, and 13 with carcinoma of larynx) 7 were smokers (2 with carcinoma of lip, 1 carcinoma of the oral cavity, and 4 carcinoma of larynx) in whom the average index of smoking amounted to 307·9. These numbers are not sufficient to draw far reaching conclusions. They show however higher tobacco consumption among women with the discussed tumours than in the control group of 1813 women (discussed in Part I of this study, where 8·4 per cent were smokers and the average index of smoking among the smokers amounted to 142·6).

Discussion

The patients with the "tobacco tract" cancer smoked markedly more than the general population represented by the control group. Is the observed connection accidental, caused by not appropriate selection of the control group? In other words—are the two groups comparable? Do they represent the same population?

Judging by Table I, the differences in the social background between patients with cancer of larynx or of the oral cavity and the control group "C" are not great since the average age, percentage of inhabitants of towns or inhabitants

of Upper Silesia as well as occupational structure of these groups are similar. The reason for the difference in structure of the group of patients afflicted by cancer of tonsils may be the small number of patients in this group. Among the patients suffering from cancer of lip, the previously discussed differences in the occupational structure and first of all a large percentage of farmers is noted. The farmers were placed in a separate group having an additional group " D " for comparison.

In none of the occupational subgroups of the control group higher percentage of smokers than 89·1, or average index of smoking higher than 512·9 were noted, and the differences in the smoking habits between the inhabitants of towns and rural areas were not great. The differences in the tobacco consumption between the control group and the patients discussed cannot therefore be explained by sampling error. The evaluation of statistical significance of these differences speaks against their accidental appearance. It should be accepted then that there exists a distinct connection between the appearance of the discussed neoplasms in Poland and tobacco smoking.

Summary

Results of a retrospective study conducted in Poland on tobacco smoking and cancer of lip, oral cavity, tonsils and larynx are presented. This study showed a distinct correlation between tobacco smoking and the above-mentioned cancers among men.

The relative chances of morbidity of smokers and the percentage of cases connected with smoking are computed.

The number of women examined was small but even among them tobacco consumption was found to be higher than on the average.

The author avails himself of the opportunity to express his gratitude to the Director of the Institute of Oncology in Gliwice—Dr. Święcki, M.D.—for encouragement and valuable advice, and to Mr. Dębogórski, Master of Laws, Director of the Polish Tobacco Industry, and to his co-workers: Mr. Trojan, Doctor of Chemistry, Mr. Trzciński, Doctor of Agricultural Sciences, Mr. Compala, Master of Chemistry, Mr. Wierzba, Master of Agricultural Sciences—for their friendliness which rendered possible this study.

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ON THE BIMODAL AGE DISTRIBUTION OF MAMMARY CARCINOMA

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In 1930—shortly after his death—a book written by von Pirquet was published. This treatise gave an analysis of the age distribution of a large number of malignant tumours, the mortality statistics having been obtained from the British Registrar-General.

One of the interesting facts presented was a bimodal type of age distribution of mammary carcinoma: it was shown that the distribution was probably built up by two separate distributions, one having its highest frequency at about 50 years of age, the second type (a "Spätkrebs") being most frequent at about 70 years.

After World War II attention to this peculiar feature was drawn again by Jacobsen (1946) and by Clemmesen (1948) who had been working with morbidity figures of the Danish Cancer Registration. From many sides confirmation of the bimodal type of age distribution was published: Anderson *et al.* (1950) gave morbidity figures from Connecticut, U.S.A., Maisin and Langerock (1955) presented graphs of Danish, Swedish and French material at the Congress of Geographical Pathology held at Washington; at the Perugia Symposium on mammary cancer Denoix (1958) showed the French data in full, while Desaive, Lavigne and Adrianne (1958) gave figures from Belgium. Further confirmation came from Ficke and Reiszig (1958) from the German Democratic Republic and from Pedersen and Magnus (1959), the latter paper being an official publication of the Norwegian Cancer Registry.

In the material of the Dutch Cancer Registry we found a bimodal type of age distribution too. Fig. 1*a* shows an age specific frequency curve of morbidity, and Fig. 1*b* presents an age specific curve of mortality, computed from data of the Central Bureau of Statistics. These curves run upwards steeply but show a diminution of this trend between the 45th and 55th year, namely in the period of onset of the menopause. This phenomenon could be interpreted by assuming that at menopausal age the curve of the first kind of mammary cancer (that of reproductive age) decreases sharply, while the curve of the second kind (the "Spätkrebs") has not increased so much that it already compensates for that decrease.

However, the typical bimodal age distribution is not found in every statistical material. The figures of Phillips and Owchar (1957) from eight countries are not considered here, because they have been related to a standard population

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from abroad (Canada). But the curves which can be constructed with the figures of Stocks (1959) show that in the metropolitan areas of the U.S.A. no significant retardation of the steeply increasing curve of age specific morbidity is seen between the age of 45 and 55 years, in contrast to the Scandinavian countries (Fig. 2). We shall return to a possible explanation of this fact later.

Several authors have been wondering what this bimodal age distribution actually means. Can we speak of two types of mammary cancer ? And in which respect do these types differ ? Is their cause also different ?

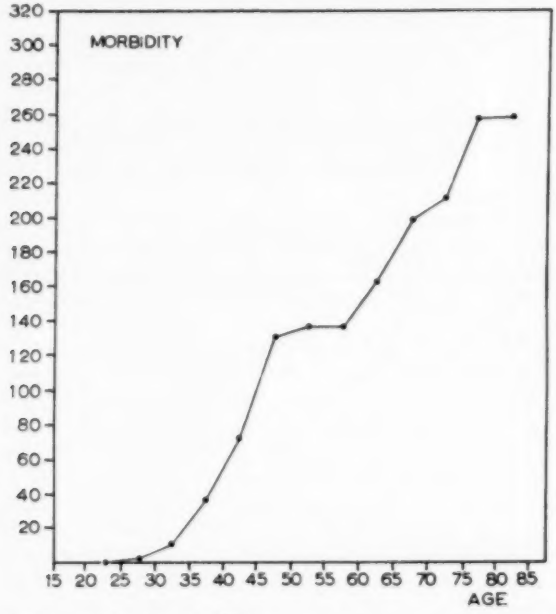

FIG. 1*a*.—Age-specific morbidity (per 100,000 women) of mammary carcinoma in the Netherlands. 3081 cases diagnosed during 1957 and 1958 and collected by the Central Cancer Registry, Amsterdam. It is estimated that about 70 per cent of all cancers are being registered at present ; no correction for this has been attempted. Population figures supplied by the Central Bureau of Statistics, The Hague (population at December 31, 1956).

Maisin and Langerock (1955) made some suggestions as to the nature of the two types. They mentioned *inter alia* that from animal experiments evidence had been obtained that in the genesis of mammary cancer of older age an adrenal dysfunction played a part. Their compatriots Desaive, Lavigne and Adrianne (1958) went a step further at the Perugia Symposium, postulating without any reserve that the type of mammary carcinoma of reproductive life was due to a dysfunction of the ovaries, and the postmenopausal type to a dysfunction of the adrenals.

Ficke and Reiszig (1958) were more cautious, but they too drew attention to the decreasing incidence of the younger type of mammary cancer when the

oestrogen production by the ovaries ceases. The curve of the older patients should be the result of a compensating oestrogen production by other endocrine glands. It is known that the adrenals may produce these substances.

Such a hypothesis is not unattractive, for animal experiments have shown that oestrogens—although physiological substances—may under certain conditions have a carcinogenic effect on the mammary gland (Mühlbock and Boot, 1959).

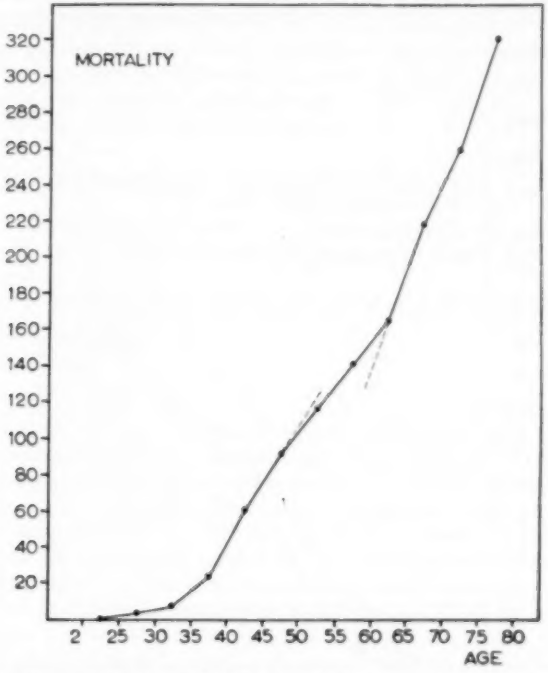

FIG. 1*b*.—Age-specific mortality (per 100,000 women) of mammary carcinoma in the Netherlands. 3191 death certificates registered during 1957 and 1958 by the Central Bureau of Statistics, The Hague. *N.B.*: It is for the greater part about other women than in the graph of morbidity. The anomaly in the curve between 45 and 55 years is less than in the curve of Fig. 1*a* probably due to the longer and inconstant period between onset and registration of the malignancies.

In laboratory animals early bilateral adrenalectomy is equally effective as early castration in lowering the incidence of spontaneous mammary carcinoma (Shimkin and Wyman, 1945). There are many indications that the therapeutic significance of bilateral ovariectomy and adrenalectomy is based on the elimination of oestrogens. If it should be possible to distinguish by clinical or laboratory means between ovarian and adrenal oestrogens the hypothesis could be tested that the two types of mammary cancer may be related to a dysfunction of the ovaries and the adrenals respectively.

Cytological work of Bruinsma and de Waard (1959) seems to enable us to distinguish between these two types. These authors, starting from epidemio-

logical considerations concerning endometrial cancer, wondered whether post-menopausal oestrogen production perhaps would occur in patients with diabetes mellitus, obesity and essential hypertension. A group of 42 diabetics showed indeed a significant higher number of vaginal smears characteristic of oestrogenic activity than did a control group. The difference between these groups was owing to the diabetic patients with obesity and/or hypertension. In the case of

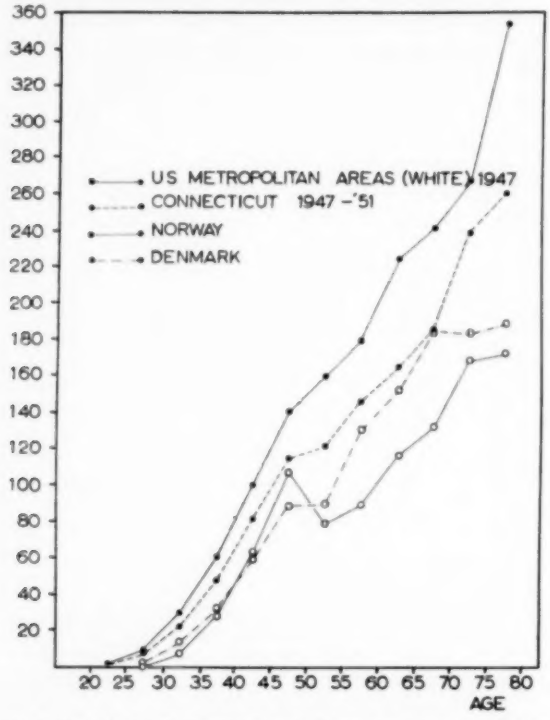

FIG. 2.—Age specific morbidity of mammary carcinoma per year per 100,000 women, after Stocks (1959, Table 4).

patients with obesity and/or hypertension without diabetes oestrogenic smears were frequently encountered too. Because of the limited number of diabetic patients without obesity or hypertension it was impossible to draw any conclusions about a possible difference between these patients and the control group. Of significance were the findings of oestrogenic smears in 17 castrated diabetics. The authors concluded that the oestrogens responsible for these effects were probably of adrenal origin.

De Waard and Baanders (to be published) made a cytological investigation of the urinary sediments of more than 100 women over 55 years of age from the population of Utrecht, Holland, who had been invited to serve as a normal control group in a genetic investigation. There exists a cytological parallelism between

the vaginal smear and the urinary sediment (Lencioni, 1953), permitting endocrine evaluation of the latter after staining with Shorr's trichrome stain. The results of this investigation confirmed and completed those of Bruinsma and de Waard (1959) by showing clearly the influence of hypertension and obesity on the frequency of oestrogenic pictures in women with ovaries as well as in those without. Moreover, a study was made of glucose tolerance in the non-obese, non-hypertensive subjects. It was shown that the few women of this subgroup presenting a certain oestrogenic activity had a decreased glucose tolerance. The authors concluded that the combined groups of postmenopausal women with obesity, hypertension and a decreased glucose tolerance contained all those exhibiting oestrogenic activity cytologically. This condition of continuous hormonal activity was called : adrenal oestrus.

PLAN OF STUDY. MATERIAL AND METHOD

Based on this cytological evidence for adrenal oestrus and on the epidemiological fact of a bimodal age distribution of mammary cancer the following plan of study was made :

Of a number of patients with mammary carcinoma we investigated :

Weight and height.
Blood pressure.
Glucose tolerance.

If obesity, hypertension or a decreased glucose tolerance were present it was assumed that adrenal oestrogen would have been present which could have promoted the carcinogenesis in the mammary gland. If none of these pathological signs was found it was supposed that any carcinogenic influence of oestrogens would have come from the ovaries. Of the " adrenal " as well as of the " ovarian " type of patients with mammary cancer an age distribution could be made ; if these age distributions coincided with the distributions found by epidemiologists in their morbidity statistics an argument would be provided for the correctness of the hypothesis.

The material obtained from 108 patients was collected in six different hospitals. The patients belonged for the greater part to the population of two large towns. Only cases of recent onset (less than six months between first diagnosis and our investigation) in which the malignancy had been confirmed by the pathologist were studied. Cases with distant metastases and those who were or had been treated endocrinologically (with hormone preparations or by surgical intervention) were omitted.

The criteria for classification according to relative weight, blood pressure and glucose tolerance were the following : a body weight more than 25 per cent above Ideal Weight was called obesity ; we used the Table of Ideal Weights given by Bøe, Humerfelt and Wedervang (1957). In laying down the criteria for hypertension we evaluated both the systolic and the diastolic pressure. In the first place the lead given by the Expert Committee on Cardiovascular Disease and Hypertension of the World Health Organisation (1959) was followed, drawing a dividing-line between normal and abnormal blood pressure at 160/95 mm. Hg. This dividing-line has been shown by Morrison and Morris (1959) not to be a purely arbitrary one. However, we considered it justifiable to include as

hypertensive cases those with a diastolic pressure of 100 mm. Hg or more if their systolic pressure was at least 150 mm. Hg, and those with a systolic pressure of 170 mm. Hg or more irrespective of diastolic pressure.

The glucose tolerance tests were carried out either pre-operatively or more than 14 days post-operatively in order to avoid the effect of surgical stress. The amount of glucose given was 50 g. by mouth ; the blood sugar estimations were performed in 73 cases according to the method of Hagedorn and Jensen and in 35 according to Folin and Wu.* In judging the blood sugar curves attention was given to both components composing a diabetic type of curve separately : 1. blood glucose level increasing too much, 2. level not decreasing to original (fasting) value within 2 or $2\frac{1}{2}$ hours. Ad 1. we considered the level to be pathological, if at least two values above 180 mg. per cent or one value above 190 mg. per cent was reached. Ad 2. we fixed a dividing line between normal and abnormal after making frequency distributions of the difference between the 2- or $2\frac{1}{2}$-hour values and the fasting values. It was seen that also at higher ages a decrease to below the fasting value often took place, and that a suitable boundary between normal and abnormal was to be found in the region of 2- or $2\frac{1}{2}$-hour levels lying 10–20 mg. per cent above the fasting values. We chose finally as the dividing line : 20 mg. per cent above the fasting value, considering curves with differences of 15–20 mg. per cent dubious ones.

RESULTS

Based on the above criteria we divided the patients into two groups : one without any pathological feature of the triad—obesity, hypertension or a decreased glucose tolerance, the second with at least one of those features. Three women who were not obese nor hypertensive, but who exhibited a dubious blood sugar curve were classified separately in Table I and omitted from Fig. 3 which thus presents the result of our separation of 105 cases. In this figure which is a graphical condensation of Table I two frequency distributions are seen with modes in the 45–50 and the 60–64 age classes respectively, which show a striking similarity to the distributions expected by epidemiologists. This does not prove that our hypothesis is correct, but it gives definite support to it.

An important question which has to be investigated is : does one in separating any female population according to the presence or the absence of obesity, hypertension and decreased glucose tolerance perhaps find two age distributions similar to the distribution of mammary cancer patients ? It is well known that the features of this triad are much more frequent later in life than in the reproductive period.

We have investigated this point with care, making use of data of a statistical-genetic study designed by two of us (not yet published). The control group needed for this study was obtained by taking 1000 cards of women out of the files of the Population Registry of the town of Utrecht (250,000 inhabitants). This was done at random apart from a certain age distribution. These women were invited to an interview about cancer in their families, together with a brief physical examination including measurement of weight, height, blood pressure and examination of the urine. Almost 60 per cent of these women co-operated ;

* As judged by frequency distributions of the fasting levels the values obtained by the former method are about 5 mg. per cent lower than those obtained by the latter.

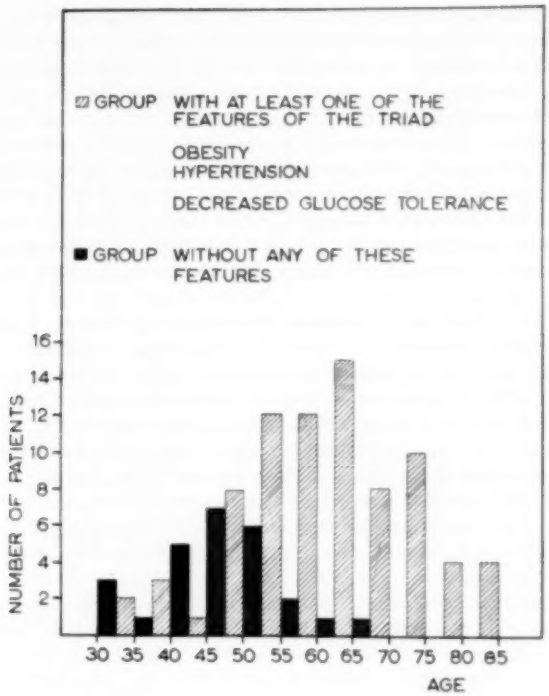

FIG. 3.—Separation of 105 cases of mammary carcinoma into two groups (see text).

TABLE I.—*Presence of Obesity, Hypertension and Decreased Glucose Tolerance in 108 Patients with Mammary Carcinoma*

Age	No pathological features	Obesity only	Hypertension only	Obesity + hypertension	Decreased glucose tolerance only	Obese + decreased glucose tolerance	Hypertension + decreased glucose tolerance	Obesity + hypertension + decreased glucose tolerance	Glucose tolerance dubious	Total
30–34	3	1	—	—	1	—	—	—	—	5
35–39	1	1	—	—	1	1	—	—	—	4
40–44	5	1	—	—	—	—	—	—	—	6
45–49	7	2	1	1	—	1	1	2	—	15
50–54	6	1	2	3	3	2	1	—	1	19
55–59	2	1	—	2	3	2	3	1	1	15
60–64	1	3	—	1	3	2	2	4	—	16
65–69	1	1	2	1	—	2	1	1	1	10
70–74	—	2	—	—	2	1	2	3	—	10
75–79	—	1	—	2	1	—	—	—	—	4
80–84	—	—	—	—	2	1	1	—	—	4
	26	14	5	10	16	12	11	11	3	108

an analysis of their addresses revealed that the living quarters of the higher educational classes were represented slightly better than the other ones. Making a comparison with a group of mammary cancer patients this does not seem to be a draw-back, because it is known that patients with this type of cancer constitute also a slight selection among the higher educational population groups.

The investigation provided us with the data of 571 women. They were divided into two groups according to the same criteria of obesity and hypertension as the 108 patients with mammary cancer (it was impossible to perform a glucose tolerance test in all of them). In view of the shape of the histograms of Fig. 3 we were interested in the question whether obesity and hypertension (occurring singly or combined) were more frequent in the postmenopausal cancer patients than in the postmenopausal controls. Table II shows that this was found indeed. Within both groups there exist small differences between the age classes which are, however, far from significant (χ^2-test). Thus it is permissible to combine the different age classes above 55 years and to summarize as follows : in the mammary cancer group 42 of 59 women are obese and/or hypertensive, that is 71 per cent. In the control group 174 of 322 women have obesity and/or hypertension, that is 54 per cent. Although this latter percentage is high too, the difference from the mammary cancer group is significant (hypergeometrical distribution, $P < 0{\cdot}02$). If the criterion for hypertension is modified in such a way that only blood pressures of at least 170 mm. Hg systolic fall into the pathological group, the difference between the cancer group and the control group becomes even greater ($P < 0{\cdot}01$).

TABLE II.—*Relative Frequency of Obesity and Hypertension (Occurring Singly or Combined) in Mammary Cancer Patients and Controls Over 55 Years of Age*

		Age						
		55–59	60–64	65–69	70–74	75–79	80–84	Total
Mammary cancer group	Number of women	15	16	10	10	4	4	59
	Number of women with obesity and/or hypertension	9	12	8	8	3	2	42
Control group	Number of women	78	77	67	45	36	19	322
	Number of women with obesity and/or hypertension	40	45	38	21	18	12	174

Thus the conclusion seems to be justified that mammary carcinoma after the menopause has a preference for the obese and the hypertensive, occurring almost exclusively in women with obesity and/or hypertension and/or a decreased glucose tolerance, conditions which are associated with oestrogenic activity of adrenal origin.

DISCUSSION

The direct evidence of a connection between adrenal oestrogen production and a decreased glucose tolerance in older people not yet being amply present, some indirect evidence may be wanted.

It is known that older patients with diabetes mellitus with or without obesity who do well on a dietary regime alone or in conjunction with oral administration of sulfonylurea derivatives (like tolbutamide, etc.) produce insulin in reasonable amounts ; their diabetes is considered a " Gegenregulationsdiabetes " (Bertram, Bendfeldt and Otto, 1956). Those who are of the opinion that the adrenals play a part in this " Gegenregulation " (Bastenie, 1956) find support in the observations of Szenas and Pattee (1959) that the glucocorticoid production in obesity is increased. This influence of overweight is also to be gathered from the statistical data of Borth, Linder and Riondel (1957). Moreover the decreased glucose tolerance which is so often found in obese subjects is of the G.I.T.T.-positive type, pointing to increased secretion of glucocorticoids too (Arendt and Pattee, 1956).

There exists a certain parallelism between the production of glucocorticoids and adrenal oestrogens : operative stress or injection of ACTH increases the urinary excretion of both types of steroids (Décourt *et al.*, 1951 ; Bulbrook *et al.*, 1958 ; Brown, Falconer and Strong, 1959) and cortisone therapy inhibits the production not only of glucocorticoids but also of adrenal oestrogens (Smith and Emerson, 1954 ; Block, McCarthy and Vial, 1959*b*).

We are not of the opinion that every curve revealing decreased glucose tolerance is of necessity a reflection of an increased secretion of glucocorticoids (it would have been preferable if also glucose-insulin tolerance tests of our patients could have been made). However, it seems reasonable to assume with French gerontologists that at older age in the larger part of these curves the adrenal cortex plays a part (Binet and Boulière, 1955), and such an assumption already satisfies the epidemiologist.

If our observations can be confirmed by others, a definite perspective regarding mammary cancer is looming up. In the first place the indications in the treatment of metastasized mammary carcinoma could get a more theoretical basis. In judging the results of different hormonal kinds of therapy the type of cancer (" ovarian " or " adrenal ") could be included in the considerations. Our curve of the " ovarian " type (Fig. 3) fits well in with the observations of Dao and Huggins (1957) that after 54 years of age bilateral oöphorectomy is seldom indicated. It could be found that young women with obesity and/or hypertension are not helped sufficiently by castration alone but that in them at an early stage the elimination of adrenal oestrogens must be advocated, either by surgical means or by suppression of pituitary ACTH with corticosteroids. Finally the fact could be understood why some patients who did not react favourably to surgical castration were subsequently greatly helped by adrenalectomy (Block *et al.*, 1959*a*), the poor castration response not being based on hormone independence but on the fact that the ovaries were inactive in contrast to the adrenals.

But of no less significance seems to us the possibility for more insight into some aetiological factors of mammary carcinoma. We venture to present the following hypothesis :

It is well known that heredity and environment play a part in the genesis of mammary cancer. Concerning the environmental factor(s) it must be stipulated that this carcinoma has a preference for the higher social classes (Stocks, 1955) and that it is seen more frequently in Western countries than in peoples of Asia and Africa (Segi *et al.*, 1957 ; Oettlé and Higginson, 1958).

It is also well known that the disease entities of the triad obesity, essential hypertension and diabetes mellitus (" diabète gras ") are more frequent in the materially blessed peoples of Western countries than in these African and Asian peoples (de Langen, 1958; Smirk, 1949). Further, obesity, hypertension and diabetes have also a hereditary aspect.

We suppose that the phenomenon of adrenal oestrus on account of its association with obesity, hypertension and decreased glucose tolerance (not only in statistical but probably also in pathophysiological respect) has also a hereditary and an environmental aspect, and that these aspects in turn determine the hereditary and the environmental aspects of the " adrenal " type of mammary carcinoma.

With this hypothesis the peculiar feature shown in Fig. 2 could be explained why the bimodality of the age-specific frequency curve of mammary cancer is not pronounced in the metropolitan areas of the U.S.A. in contrast to the Scandinavian countries. In these parts of the U.S.A. the Western technical civilization finds its summit, with all the life habits (nutrition *inter alia*) inherent to it. If diabetes, obesity and hypertension and the phenomenon of adrenal oestrus begin to occur a few years earlier in life and if their frequencies increase with age somewhat stronger than elsewhere in the Western world, the " adrenal " group of mammary cancers will overlap the " ovarian " group almost completely masking the decrease of the latter group at menopausal age.

This environmental aspect of the older type of breast cancer may also explain certain facts mentioned by McMahon (1957), himself a critic of the bimodal type hypothesis, namely, (1) the difference in mortality trends during the 20th century between mammary cancer among pre- and postmenopausal women respectively, (2) the shift in the incidence break of the age specific morbidity curves of breast cancer in Connecticut, (3) the differences between the curves of Danish urban and rural areas.

In contrast to the life habits of Western countries we know of peoples with ways of living in which diabetes, obesity and hypertension are relatively unknown. Perhaps by changing our nutritional habits we might be able to reduce not only the incidence of these diseases but also that of adrenal oestrus and of the " adrenal" type of mammary carcinoma.

SUMMARY

Morbidity and mortality statistics suggest that the population of mammary cancer patients in Western countries is composed of two populations, each with its own age distribution having their highest frequencies about 48 years and 65 years of age respectively. Statistics from the Netherlands show this bimodal distribution too.

In trying to find a possible basis for this phenomenon the hypothesis is discussed that the type of mammary cancer of reproductive age is caused by an ovarian dysfunction and the type of older age by an adrenal dysfunction. This hypothesis is tested by applying epidemiologically the fact established by endocrine cytology that patients with obesity, essential hypertension and/or a decreased glucose tolerance often show signs of oestrogenic activity which are very probably of adrenal origin.

The results of this application seem to be in agreement with the mentioned

hypothesis. The possible significance of these facts for more insight into the hereditary and the environmental aspects of mammary cancer is discussed.

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THE FREQUENCY OF CANCER IN DIABETES MELLITUS

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THIS paper is concerned with the problem of whether the incidence of malignant diseases in diabetics is significantly different from that in non-diabetics. While the material of autopsies from the Pathology Department of Bristol University and the clinical material from the Bristol Royal Infirmary confirm the negative association between the two diseases which has been the subject of previous investigations, a critical examination reveals the new fact that such a negative association would seem to apply more to females. It is shown that the explanation of a negative association between malignant tumours and diabetes is in agreement with what has emerged so far about a hormonal implication via the disturbance of the glucose metabolism in both diseases.

Section 1

The results of an examination of post mortem records in the Pathology Department of the University of Bristol are shown in Table I.

TABLE I.—*Mortality Due to Malignant Tumours and Diabetes from the Post Mortem Records in the Pathology Department, University of Bristol*

Total number of post-mortem records in the Bristol Pathology Department	6317
Number of malignant tumours among them	1212
Number of diabetics among the 6317 records	189
Number of coincidences of diabetes and cancer	19
Percentage of malignant cases in the total of 6317 cases	19·2
Percentage of malignant tumours among non-diabetics (1193/6128)	19·5
Percentage of malignant tumours among diabetics	10·1

Table II gives the distribution of post mortems (P.M.'s) according to age and sex for the total of P.M.'s, the cases of malignant tumours, of diabetes, and the cases of joint occurrence of malignant tumour and diabetes.

Particular attention is drawn to the fact that the age distribution (total) of diabetics appears to be, by and large, a good replica of that for diabetes morbidity in the general population. It shows the characteristic increase in diabetes morbidity between the ages 50–80 with a pronounced peak between 60 and 70 just like the diabetes morbidity curve in the general population.

On the face of it, the diabetics appear, according to Table I, less susceptible to the malignant disease, the difference being about 9 per cent. There is nothing in the age distributions as displayed in Table II which would suggest a statistical bias to be responsible for that difference. Since we are dealing here only with samples—although fairly large ones—a difference of this type is to be tested for significance. The significance test can be carried out in different ways. We

TABLE II.—*Age and Sex Distribution of the Post Mortem Cases Summarized in Table I*

Age group	Total of P.M.'s			Cases of malignant tumours			Cases of diabetes			Malignant tumour plus diabetes		
	M.	F.	Total	M.	F.	Total	M.	F.	Total	M.	F.	Total
0–9	840	657	1497	19	18	32	2	3	5	—	—	—
10–19	99	97	196	7	8	15	0	1	1	—	—	—
20–29	149	151	300	14	7	21	2	3	5	—	—	—
30–39	186	185	371	23	18	41	2	5	7	—	—	—
40–49	367	281	648	75	49	124	9	8	17	1	2	3
50–59	637	369	1006	166	114	280	12	24	36	3	1	4
60–69	717	450	1167	224	119	243	17	45	62	2	3	5
70–79	503	351	854	176	96	275	13	32	45	4	2	6
80–89	133	120	253	47	28	75	4	7	11	1	—	1
90–99	10	14	24	2	2	4	—	—	—	—	—	—
100 +	—	1	1	—	—	—	—	—	—	—	—	—
Totals	3641	2676	6317	753	459	1212	61	128	189	11	8	19

may first regard the percentage of malignant tumours among the total of autopsies—6317—as the statistical population percentage of malignant disease, and the number of diabetics—189—as a random sample of the total. We then have, for the standard error of the population percentage in such a sample of 189 specimens

$$\sigma = \sqrt{\frac{19{\cdot}2 \times 80{\cdot}8}{189}} = 2{\cdot}86\%$$

and $3\sigma = 8{\cdot}56$ per cent added and subtracted from the basic percentage, 19·2, gives 27·76 per cent and 10·64 per cent respectively as the upper and lower limits within which we could expect the percentage of malignant tumours in a sample of 189 specimens to lie. The observed percentage of 10·1 among the 189 diabetics lies beyond the 3σ limit and is, therefore, to be regarded as highly significant of an assignable cause.

The same answer is obtained if we estimate the population percentage on the basis of the sample percentage, by means of the chart on page 61 of 'Statistics of Therapeutic Trials' (Herdan, 1955). Entering the chart with $p = 10{\cdot}1$ on the upper horizontal scale and projecting the crossing point of the vertical with the curve $n = 189$ to the left (or right), we read there the difference between the upper limit for the population probability p_u, and p as slightly less than 8, and obtain, therefore, the upper limit p_u as slightly less than 18, which is significantly smaller than the observed figure, 19·1 per cent.

A second possibility is to regard both the percentage of malignant tumours among the total of P.M.'s and that of coincidence cases of the two diseases as samples from the statistical population of P.M.'s in the wider sense, say in the whole of the country, and determine the significance between the two. The result is essentially as before.

Section 2

It is now important that there is a remarkable stability of the figures obtained for the malignant disease, in general, and among diabetics, not only *within* a country, but *between* countries, and significance tests could also be carried out

on the percentages of the same description but in different countries. That is, we could compare the percentages of malignant diseases in, say, England and Germany, and the percentage of coincidence cases in these two countries.

The justification for such a procedure—which is rather important both from the theoretical and practical angle as will be explained below, can be seen from Table III which gives relevant numbers as in Table I for six investigations of that kind.

TABLE III.—*Mortality Due to Malignant Tumours and Diabetes from German Post Mortem Records*

	Kruger (1940)	Mallory In.	Eskuchen (1947)	Welsch (1952)	Eichler (1954)	Werner (1953)	Rockstroh and Schröter (1960)
Post mortems	5,844	—	10,948	13,546	19,242	25,147	22,971
Malignant tumours	731	—	1,894	2,271	3,524	4,989	4,142
Diabetics	122	307	236	402	637	705	351
Malignant tumours and diabetics	10	24	21	40	59	50	31
Diabetics as percentage of total P.M.'s.	2·09	—	2·4	2·97	3·3	2·8	1·53
Malignant tumours as percentage of total P.M.'s.	12·15	—	17·3	16·77	18·3	19·8	18·1
Malignant tumours as percentage of non-diabetics	12·7	—	17·7	17·3	18·95	20·4	18·3
Malignant tumours as percentage of diabetics	8·2	8·4	8·3	9·95	9·27	7·1	8·8
Time of publication	1940	1944		1952	1954	1955	1959

We shall consider as a particular case the last of these investigations which does not differ essentially from the others in its results. On the basis of 22,971 P.M. records from the University Clinic of Halle a/S the percentage of malignant cases resulted as 18, remarkably close to the figure for Bristol, 19·2. The percentage of coincidence cases of diabetes and malignant disease was for Halle 8·8 per cent, again remarkably close to the figure for Bristol, 10·1 per cent. The significance test shows that we may regard the basis of the two investigations as one statistical population of P.M.'s in which the percentages of tumours, 18 and 19·2 differ by not more than could be accounted for by random sampling from that one population. The same applies to the percentages of coincidence cases, 8·8 and 10·1 per cent.

To counter the possible objection that the negative association between malignant disease and diabetes is only apparent and due to the fact that the diabetic does not live long enough to be attacked by malignant disease, we give below the age distribution of the coincidence cases which shows the coincidence cases as not being different in age distribution from the total of cancer cases.

The same is, by and large, true for the German coincidence cases. Both malignancy and diabetes and also the coincidence cases, increase with age and then decrease again. The peak of the age curve in Germany lies between 55 and 65 years for malignancy and for the diabetics between 61 and 70 years, and for the coincidence cases between 71 and 80 years. This means that today diabetics reach a sufficiently high age to be exposed to the risk of the malignant disease like other parts of the population.

TABLE IV.—*Age, Sex and Site for the 19 Coincidence Cases of Cancer and Diabetes Mentioned in Table I*

Site	Sex	Age
Breast	F.	62
	F.	60
	M.	80
Gall bladder	F.	47
Kidney	M.	74
Larynx	M.	83
Lung	M.	57
	F.	47
	M.	46
	M.	75
Pancreas	M.	65
	F.	51
	M.	58
Rectum	M.	73
	M.	70
Stomach	F.	74
	F.	65
Leukaemia	F.	73
Lymphocarcinoma	M.	64

Although the difference between the Bristol percentages and those of Halle of the same denomination are not statistically significant, yet one might be somewhat uneasy about what looks like a slight systematic increase in the Bristol percentages: 19·2 against 18 per cent for malignant cases, and 10·1 against 8·8 per cent for coincidence cases. However, the explanation may lie in the change of diabetes incidence with time. As is well known, diabetes is, in general, on the increase in both England and Germany and so is cancer of certain sites. In spite of the agreement between the Bristol figures and those from Halle a/S there is a difference in the sex distribution between the two samples. For Halle the relation between M. and F. among the diabetics is 0·78 to 1 and that for the coincidence cases 0·82 to 1. For the malignant tumours without diabetes the relation is 1·33 to 1. The corresponding figures for Bristol are 0·48 : 1, 1·38 : 1 and 2·24 : 1. The statistical influence of the striking difference in sex distribution for tumours and diabetes upon the coincidence rate is discussed in Section 5.

Regarding the relation of the analysis of post mortem records to mortality statistics of the whole population in England and Wales, the following figures were obtained for 1950:

	Deaths as percentage of all deaths	(%)
Cancer		
M.	0·170	17
F.	0·172	17 approx.
Diabetes		
M.	0·00468	0·5
F.	0·00989	1·0

Although the percentage of cancer cases among the P.M.'s is in fairly good agreement with that in the total population—19 as against 17 per cent—the percentage of diabetics among the P.M.'s, approximately 3 per cent, is rather higher than the percentages in the total population, 0·5 for males and 1·0 per cent for females,

which might conceivably have been responsible for a *higher* percentage of coincidence cases, but not for the actually observed *lower* percentage. However, attention is drawn to the fact that the relation of diabetic M. and F. in the population is almost precisely that for the diabetics among the P.M.'s. We have

$$61 \text{ M.} : 128 \text{ F.} = 1 : 2$$

and in the population, we have the diabetes percentage of all deaths

$$0{\cdot}5 \text{ M.} : 1{\cdot}0 \text{ F.} = 1 : 2.$$

Section 3

The stability of the corresponding percentages in different investigations is so remarkable that it calls for comment from both the medical and statistical angle. It is often maintained that P.M.'s cannot be regarded as a random sample of the mortality in a given country, since there is no definite rule as to which cases are to be subjected to autopsy. It largely depends on the preference which the consultants in question have in this respect, and this may be different at different times according to which type of disease is specially in the foreground of medical interest. This is then adduced as a reason for the doubtfulness of comparability between mass results based on P.M.'s.

While it is readily admitted that the basis of true comparability of statistical results is that they were obtained in the same manner, yet to overdo this and restrict the possibility of comparison in this way would be against the very nature of statistics. Statistics is a method to be applied where the strict requirements of laboratory experimentation cannot be obtained. In observation of masses of events which is the very essence of statistics, it is just the impossibility of following each individual case which makes statistical methods necessary. That in spite of the lack of complete comparability between mass results there should be a certain stability is the very basis for the conception of medical statistics as conceived by Dr. Farr.

Applying these considerations to P.M.'s, we must, on the basis of the present investigation, arrive at the conclusion that in spite of the P.M.'s at different hospitals being carried out by different persons with different points of view, a sufficiently great number of these autopsies—provided only that there is no true, i.e. intentional, bias of the investigator—can be regarded as a sample of the population of P.M.'s. Rightly understood, a conclusion of this kind expresses in words what must be the basis of any medical inference about a certain amount of protection from malignant diseases in diabetics. As a biological or pathological fact, it must apply to mankind in general, provided only that the living conditions, etc., are comparable.

However, in spite of giving, by and large, a fair picture of the relations of the rates in the population for the two diseases in question, we cannot, in the strict sense of the term, regard a given mass of P.M.'s as a random sample of the population mortality, but the stability of proportions we have noticed among the different *masses of P.M.'s* justifies regarding these masses as *representing a statistical population in its own right*. What it loses by not being strictly a random sample of population mortality it gains by the greater reliability of diagnosis and by the completeness of the morbidity picture which it affords.

The difference between the distribution of deaths from certain causes in the general population and in the population of P.M.'s is readily admitted, but it does

not matter for our argument, since the percentages which we compare, i.e. those of malignant tumours and diabetes, are both from the same population of P.M.'s. The difference from the general population influences therefore both percentages in the same way.

It should also be noted that we are not concerned with the percentage of diabetics among the P.M.'s, which may, and probably will, differ from that in the general population, but only with the percentage of diabetics among tumours, i.e. with the coincidence cases. Even if the number of diabetics were systematically too small compared with what it is in the general population, this need have no influence upon the number of coincidences.

Section 4

According to the literature, the clinical experience did not always seem to bear out the conclusion reached from a study of P.M.'s. Thus, Constam (1950), who gives the percentage of the incidence of malignant tumours in non-diabetics as 3·0 per cent and among diabetics as 5·1 per cent.

In order to check these findings I obtained from Bristol Royal Hospital, Royal Infirmary Branch, the corresponding data for 7 years, 1953–59, which are set out in Table V.

TABLE V.—*Morbidity Due to Malignant Diseases and Diabetes in the Bristol Royal Infirmary*

1	2	3		4	5	
		Malignant diseases registered			Patients with joint occurrence of diabetes and malignant tumour	
Year	Total number of in-patients	Number	percentage of in-patients	Total number of patients with diabetes	Number	Percentage of diabetics
1953	8,587	463	5·39	142	3	2·11
1954	9,150	409	4·47	145	6	4·14
1955	9,592	405	4·22	169	5	2·92
1956	9,651	753	7·80	171	2	1·17
1957	9,446	578	6·12	202	8	3·96
1958	9,524	587	6·16	205	4	1·95
1959	8,755	488	5·57	195	7	3·58
Totals	64,705	3,683	5·69	1,229	35	2·84

The results are most interesting in so far as they seem fully to support the conclusion obtained from our P.M. data. The incidence of malignant tumours among diabetics is considerably less than among all in-patients, the ratio between the two overall percentages, 2·84 and 5·69, being approximately 0·50 which is in good agreement with the ratio of the corresponding percentages obtained from our P.M. data, 10·1 and 19·2, which is 0·53. The percentage of tumours among non-diabetics is 5·75.

On the basis of our data, be they from P.M.'s or from clinical experience, we have therefore, no reason to doubt the significantly reduced incidence of malignant tumours among diabetics.

Section 5

Inspection of Table II with regard to the sex distribution, reveals the remarkable fact that the number of female diabetics is more than twice that of male

diabetics. This, in connection with the smaller frequency of malignant diseases in females (M. : F. = 1·64 : 1), at once suggests that the negative association between diabetes and malignant tumours may not be quite homogeneous as regards the sexes. Using the following symbols

C_T = Total of malignant tumour patients
T = Total of P.M.'s
C_D = Malignant tumours among diabetics
D = Number of diabetics
and the subscript $_m$ for male and $_f$ for female

we have for males

$$C_m/T_m = K_1 \frac{C_{Dm}}{D_m}$$

and substituting our figures

$$0{\cdot}207 = K_1 \times 0{\cdot}180$$
$$K_1 = \frac{0{\cdot}207}{0{\cdot}180} = 1{\cdot}15$$

and for females

$$C_f/T_f = K_2 \frac{C_{Df}}{D_f};$$

in figures:

$$0{\cdot}172 = K_2 \times 0{\cdot}063$$
$$K_2 = 0{\cdot}172/0{\cdot}063 = 2{\cdot}73$$

In words: for males the incidence of malignant tumours in the population of autopsies is 1·15 times that among diabetics, whereas for females the corresponding figure is 2·73; or conversely, the occurrence of malignant tumours among diabetic males is 87 per cent of that in the total autopsies population, whereas for females it is only 37 per cent.

This means that if the difference in sex distribution is duly taken into account we find that the observed overall negative association between the two diseases is, by and large, true only for the female sex. For males, the incidence of malignant diseases in the total of autopsies is not significantly different from that among diabetic autopsies (1·15 times).

Section 6.

Common to both diseases is the pathological change in glucose metabolism. In diabetes the transport of glucose through the cell membrane is slowed up. According to Rockstroh and Schröter (1960), the application of insulin causes glucose to pass at an increased rate through the membrane into the cell, where it is used for the production of energy. On the other hand, in the cancer cell, fermentation of glucose goes through the same enzymatic reaction as in glycolysis, and since the latter is activated by the application of insulin, insulin will increase an existing tumour; Rockstroh and Schröter give references for this view. Considering now that the quantity of glucose required for producing a given amount of energy by glycolysis is about 10 times that required for producing the same amount of energy by means of respiration (oxidation), they argue that it would seem understandable why a diabetic in the critical age does not suffer from metabolic upsets which would be favourable for the development of malignant tumours. The glucose is not allowed to accumulate in the cell but is used on a large scale for the

production of the energy by glycolysis because the other pathway, respiration, is much reduced in the tumour cell.

The pioneer work of Warburg on the energy-producing reactions of tumours has led to a vast amount of research on the subject. As a result, it is now clear that these reactions depend to a large extent on the metabolism of carbohydrates, and that the metabolic pathways of neoplastic cells differ considerably from those of normal cells. Tumour cells can carry out both aerobic and anerobic glycolysis but the latter process is particularly enhanced and very high rates may be manifest.

Whilst the shift of emphasis in the glycolitic and respiratory capacities of neoplastic cells from that obtaining in normal cells is well recognized, its significance has been a point of controversy for many years. Warburg (1956) considers it to be an irreversible alteration resulting from a change in the mitochondrial respiratory system. In his view, damage to the respiratory apparatus in mitochondria of normal cells is followed by a selective process favouring the survival of cells capable of increased permeation as a means of compensation. Many other investigators believe however that the process of carcinogenesis depends basically on nuclear changes, and Kit and Griffin (1958) regard primary damage to respiratory grana as being significant only if it results in nuclear alteration.

Thus in neoplasms there is a disturbance of carbohydrate metabolism localized in the tumour cells, whereas in diabetes mellitus a generalized alteration of the very same metabolic process exists. Henderson and LePage (1959) pointed out that in the presence of an unusual carbohydrate metabolism certain compounds may become essential to the growth of a tumour which would otherwise be regarded as non-essential. In their review they summarize experimental observations which could be interpreted as evidence of an avidity of tumour cells for glucose such that the normal tissues of the host are depleted of sugar. From this it seems that a neoplasm would be capable of influencing the severity of an existing diabetic state, so that the local disturbance of carbohydrate metabolism in the tumour affects the general state of carbohydrate metabolism in diabetes.

This is the reverse of the possible influence of diabetes upon the incidence of human tumours which emerges from the P.M. analysis given in Sections 1 and 2.

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EARLY (PROPHYLACTIC) OOPHORECTOMY AND ADRENALECTOMY IN CARCINOMA OF THE BREAST; AN INTERIM REPORT

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EARLY in 1953 following the pioneer work of Huggins, we began to treat selected cases of advanced carcinoma of the breast by bilateral oophorectomy and adrenalectomy. We confirmed, as have many others, that in a proportion of cases palliation may be achieved, and in a very small proportion possible prolongation of life, the longest survival time in our series being 4 years. In most cases however, times both of palliation and of survival had to be measured in months rather than years. We therefore considered the possibility that the limited benefits of hormonal surgery might be related to the advanced stage of the disease at the time of operation, and might be improved by earlier operation. We decided to put this hypothesis to the test by operating on a small number of patients with a bad prognosis as soon after the primary operation as practicable. The first patient was operated on in November 1954, and since that date there have been 10 further cases. Many years would have to pass before there could be positive evidence that the operation was of value, but evidence against the operation being of value could accumulate in a shorter time. Such evidence appears to be accumulating, and is reported in the present paper.

SELECTION OF CASES (Table I)

We have deliberately kept the numbers small. Two essential criteria of selection have been adopted. First, the prognosis must be bad, and secondly, the patients must be able to appreciate the nature of the problem, though we naturally dwelt on the hopeful aspect of what we were attempting. In all cases the husbands were also interviewed and the problem explained. In general, the policy was to perform bilateral oophorectomy and adrenalectomy within a few months of the standard breast removal and axillary clearance, and when the patients were apparently free of disease. Exceptions were Cases 1 and 4 in which there were supraclavicular glands, Case 2 in which there was a parasternal recurrence, Case 3 in which there was evidence of a paratracheal gland on X-ray of the chest, and Case 5 in which there was a minute local skin nodule. But in all these five cases the amount of growth present was minute in comparison with the cases with wide dissemination normally treated by hormonal surgery. The main pathological criterion was the finding on microscopical examination of growth in both the axillary and internal mammary glands, a finding shown by Handley (1958) to point to the very high probability that the patient will die from recurrence of the disease within 5 to 7 years of the primary breast operation. This pathological criterion was present in 8 of all 11 cases (Cases 3, 5, 6, 7, 8, 9, 10, 11).

Attempted prophylactic operations of this type must also satisfy the two humane criteria that there is substantially no operative mortality and no appreciable morbidity. In this series, there were no operative deaths ; all patients have been seen every month with Dr. John Nabarro at a special adrenalectomy clinic when their replacement therapy was regulated, but apart from this have been leading normal healthy lives.

TABLE I

Patient	Age	Date of adrenalectomy	Indication for operation	Time since adrenalectomy (months)	Result
1. Mrs. K—	43	Nov. 1954	Supra-clavicular glands	16	Died, recurrence.
2. Mrs. D—	48	Aug. 1955	Para-sternal recurrence	39	Ditto.
3. Mrs. O—	55	Sept. 1955	Invasion axillary int. mammary and paratracheal glands	56	Alive and well.
4. Mrs. N—	33	Feb. 1956	Acute pregnancy carc., invaded supra-clavicular glands	10	Died, recurrence.
5. Mrs. W—	59	Nov. 1957	Invasion axillary and int. mammary glands and minute local nodule	42	Alive, recurrence.
6. Mrs. T—	53	Dec. 1957	Invasion axillary and int. mammary glands	15	Died, recurrence.
7. Mrs. L—	36	Jan. 1958	Ditto.	28	Alive and well.
8. Mrs. H—	56	July 1958	,,	22	Ditto.
9. Mrs. M—	54	Oct. 1958	,,	19	Alive, recurrence
10. Mrs.G—	47	Jan. 1959	,,	16	Alive and well.
11. Mrs. P—	45	Oct. 1959	,,	7	Ditto.

RESULTS (Table I)

The patients are tabulated in chronological order of operation, and it will be seen that of the first 6, i.e. those with the longest follow-up, 4 have died of the disease, one is alive with recurrence, and one is alive and well just over 4½ years after the adrenalectomy. Apart from this last case, 4 other patients are alive and well, but the longest time since operation is only just over 2 years, and one patient operated on as recently as October 1958 though alive has a recurrence.

DISCUSSION

The fact that, though the follow-up is only a short one, 6 out of the 11 patients are either dead from recurrence, or though alive have recurrence, is evidence against the value of early oophorectomy and adrenalectomy in carcinoma of the breast. The evidence is not conclusive and may not be so for some years, since the patients that have died or developed recurrence may have had " hormone-independant " tumours, and some of those still alive and well may have " hormone-dependant " tumours and will continue to do well. Case 3 might well belong to the postulated latter group since not only has she remained well for 4½ years, but the paratracheal gland noted on X-ray examination of the chest has remained unchanged during this time. Against this more optimistic view however is the

fact that with the exception of Case 3, all the other 5 patients followed up for 3 years or more fall into the dead or recurrent group, and " hormone-dependant " tumours are generally thought to constitute a higher proportion than 1 in 6. But in addition to this, there was in fact evidence of " hormone-dependancy " in some of these patients. Thus in Case 2, the parasternal recurrence disappeared following the hormonal surgery, and in Case 4 the supraclavicular glands, in both cases these particular manifestations not reappearing during the terminal generalized phase of the disease. Thus, though one cannot yet be definite, one must at present unfortunately conclude that early oophorectomy and adrenalectomy are unlikely to offer a significant contribution to the problem of carcinoma of the breast.

SUMMARY

1. In 11 patients with carcinoma of the breast, oophorectomy and adrenalectomy have been performed as soon as practicable after the primary removal of the growth by standard surgical methods.

2. Though the follow-up is only a short one, 6 patients either have already died of the disease, or though alive have developed recurrence.

3. A longer follow-up will be needed before final conclusions are reached, but the interim conclusion seems justified that early oophorectomy and adrenalectomy are unlikely to offer a significant contribution to the problem of carcinoma of the breast.

I would like to acknowledge my indebtedness to Dr. John Nabarro whose close collaboration in the selection of patients for operation, the operative and post-operative management, and the follow-up, have been an essential and stimulating part of this study.

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URINARY 17-KETOSTEROIDS AND THEIR FRACTIONS IN WOMEN WITH BREAST CANCER TREATED BY ENDOCRINE SURGERY

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THE neutral 17-ketosteroids excreted in the urine of normal women are mainly metabolites of the hormones of the adrenal cortex. They can be separated by chromatography into two main fractions, those which have an oxygen atom at the 11-position in the steroid nucleus (the 11-oxy 17-ketosteroids) and those in which this function is not present (the 11-deoxy 17-ketosteroids). The 11-oxygenated steroids are mainly derived from the 21 carbon atom (C21) glucocorticoids, i.e., cortisol while the 11-deoxy compounds are for the most part excretion products of the C19 androgenic hormones. In this paper we report the results of estimating these urinary steroids in women with advanced breast cancer treated by endocrine surgery.

METHODS

Urinary 17-ketosteroids and their fractions were estimated in women with metastatic or recurrent mammary cancer treated by oophorectomy, adrenalectomy or radioactive implantation of the pituitary gland (Forrest, *et al.*, 1959). Collections of urine for assay were made while the patients were in hospital, the urine being collected in clean bottles and stored below 4° C.

Total neutral 17-ketosteroids were estimated in duplicate on 100 ml. aliquots of total 24-hour urine collections by the method advised by the Clinical Endocrinological Committee of the Medical Research Council (1951). After acid hydrolysis the free steroids were extracted with carbon tetrachloride and phenolic steroids (oestrogens) removed by an alkaline wash. The concentration of remaining neutral 17-ketosteroids was estimated colorimetrically by the Callow-Zimmerman reaction.

17-Ketosteroid fractions.—Fractionation of 17-ketosteroids was carried out on 24-hour or pooled 72-hour collections of urine by the method described in detail elsewhere (Hobkirk, 1958). After initial saturation of the urine with ammonium sulphate ($(NH_4)_2\ SO_4$) the glucuronide and sulphate steroid conjugates were extracted by shaking with ether-ethanol (3 : 1) and successively hydrolysed by limpet β-glucuronidase and hydrochloric acid at room temperature. The resulting free steroids were extracted with ether, washed with alkali and ketonic fractions prepared with Girard T reagent. These were separated into 3α and 3β hydroxysteroids by treatment with digitonin. Further fractionation of the 3α hydroxysteroids into 11β-hydroxyaetiocholanolone plus 11β-hydroxyandosterone, 11-ketoaetiocholololone, 11-ketoandrosterone, androsterone and aetiocholanolone was carried out by chromatography on paper in a ligroin-propylene glycol system.

Quantitative estimations of each fraction was carried out by a modification of the Callow-Zimmerman reaction (Wilson, 1954), absolute values for each steroid being obtained by reference to calibration curves for the pure compounds. Where amounts permitted, ultraviolet absorption curves of the chromatographic fractions in concentrated sulphuric acid yielded further proof of identity. The probable source of these fractions are shown in Table I.

TABLE I.—*Neutral 17-ketosteroid Fractions in Female Urine and their Probable Source*

Hormone	Urinary metabolite	Ketosteroid fraction	3 Hydroxyl orientation
Cortisol	11β-Hydroxyandrosterone	11-oxy 17-ketosteroids	3α fraction
	11β-Hydroxyaetiocholanolone		
11β-Hydroxy Δ^4-Androstendione	11-Ketoandrosterone		
Cortisone	11-Ketoaetiocholanolone		
	Androsterone	11-deoxy 17-ketosteroids	
Δ^4-Androstene 3, 17-dione	Aetiocholanolone		
Dehydroepiandrosterone	Dehydroepiandrosterone		3β fraction

RESULTS

Total neutral 17-ketosteroids

Adrenalectomy and oophorectomy.—In twelve post-menopausal women with breast cancer total neutral 17-ketosteroids were estimated in at least three 24-hour collections of urine before and in at least three collections made 1 to 3 months after adrenalectomy and oophorectomy. Post-operative urine was collected while patients were receiving maintenance cortisone (50 mg./day) and also during a 5-day period when they were given 120 units corticotrophin (ACTH) gel daily by intramuscular injection. Six of the twelve patients had objective evidence of regression of cancer following the operation ; in the others the course of the disease was not affected.

The excretion of neutral 17-ketosteroids in the urine was significantly depressed by surgical removal of the adrenals and ovaries (Table II—mean difference $\pm$ standard error : 1·4 $\pm$ 0·6 mg./24 hr ; $P < 0{\cdot}05$). The mean fall in the six patients whose cancer regressed was identical to that in those who did not benefit. In neither group of patients did ACTH produce a significant increase in ketosteroid excretion (Table II—mean difference in responding patients—0·4 $\pm$ 0·6 mg./day ; in non-responding patients + 0·2 $\pm$ 0·5 mg./day. Fig. 1).

TABLE II.—*Mean Excretion of Total Neutral 17-ketosteroids (mg. per day) in 12 Patients with Breast Cancer Before and after Adrenalectomy and Oophorectomy*

	Cancer regressed (6 patients)	Cancer not improved (6 patients)	Total
Before adrenalectomy—no cortisone	4·5	4·3	4·4
After adrenalectomy—cortisone 50 mg. per day	3·1	2·9	3·0
After adrenalectomy—cortisone 50 mg. ACTH 120 units	2·7	3·1	2·9

Pituitary implantation.—Serial daily estimations of total neutral 17-ketosteroids were made in eighteen post-menopausal women with breast cancer before

and after implantation of the pituitary with Yttrium-90. Urine was collected post-operatively while patients were receiving maintenance cortisone 50 mg./day.

The pre-operative results are the means of three to six determinations while the post-operative means are those of all estimations carried out within 3 months of implantation. Nine patients had regression of cancer as a result of pituitary destruction. Neither in them nor in the other nine patients whose cancer failed

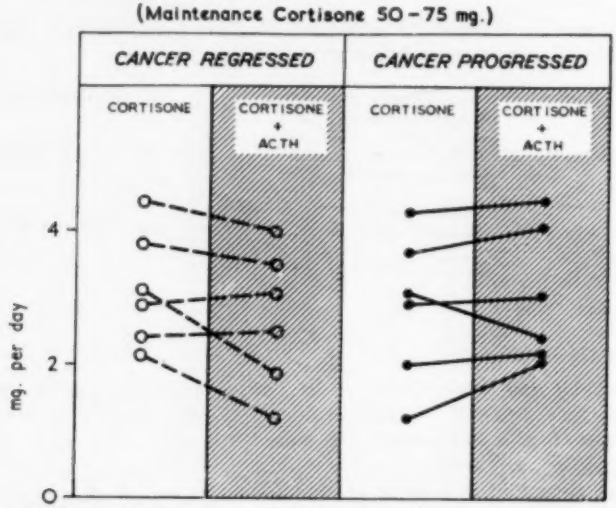

FIG. 1.—Effect of ACTH (120 units per day for 5 days) on urinary neutral 17-ketosteroids following adrenalectomy and oophorectomy in twelve patients with advanced breast cancer.

to respond, did pituitary implantation lead to significant reduction of total 17-ketosteroid excretion (Table III ; Fig. 2).

TABLE III.—*Mean Excretion of Total Neutral 17-ketosteroids (mg. per day) in 18 Patients Before and After Implantation of the Pituitary with Yttrium-90*

	Cancer regressed (9 patients)	Cancer not improved (9 patients)	Total
Before implant—no cortisone	3·5	3·6	3·5
After implant—cortisone 50 mg. per day	3·2	3·9	3·5
Mean difference	−0·3±0·4	+0·3±0·9	—
P	>0·4	>0·7	—

Neutral 17-ketosteroid fractions

Pre-operative estimations.—Neutral 17-ketosteroid fractions were estimated on 3-day pools of urine in twenty-two patients with advanced breast cancer before treatment by endocrine surgery. Five of the patients had functioning ovaries at the time of estimation (marked with P. Fig. 3). In two patients only the total 11-deoxy and 11-oxy fractions were estimated ; in the other twenty patients full

fractionation was carried out. The excretion of these fractions in twelve patients who had subsequent regression of cancer was quantitatively similar to that in ten patients who had no response to treatment (Table IV ; Fig. 3). The means of

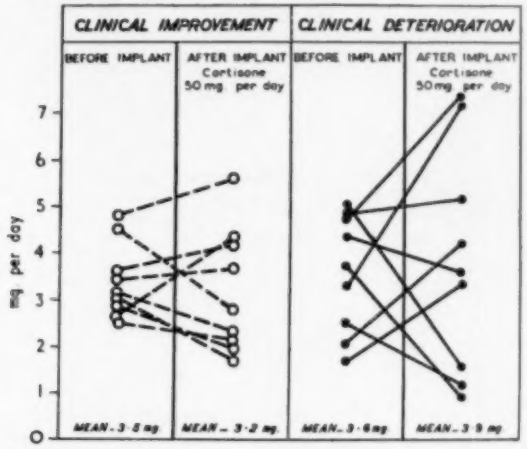

FIG. 2.—Neutral 17-ketosteroids in the urine of eighteen patients with advanced breast cancer before and after implantation of the pituitary with Yttrium-90.

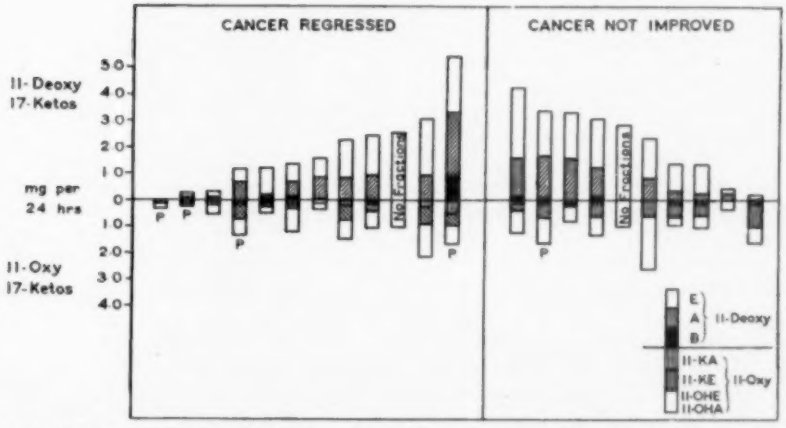

FIG. 3.—Urinary 17-ketosteroid fractions in twenty-two women with advanced breast cancer before endocrine surgery has been performed.

the two groups of patients were compared by Students " t " test using both the arithmetic values and the ($\times$ 10) logarithms of the outputs of ketosteroid fractions. No significant difference was revealed (Table V). The ratio of 11-deoxy to 11-oxy 17-ketosteroids was also unrelated to the response to endocrine surgery (Table VI).

TABLE IV.—*Mean Excretion of Urinary 17-ketosteroid Fractions in 20 Women with Breast Cancer Before Endocrine Surgery. Two Patients in Whom Only Total 11-oxy and 11-deoxy 17-ketosteroids were Estimated are Not Included in this Table but Are Included in Tables V and VI and Fig. 3*

Response to endocrine surgery	11-oxy 17-ketosteroids				11-deoxy 17-ketosteroids			
	11-OH E 11-OH A	11-KE	11-KA	Total	E	A	β fraction	Total
Cancer regressed (11 patients)	0·52	0·31	0·10	0·93	0·93	0·71	0·12	1·76
Cancer not improved (9 patients)	0·74	0·44	0·08	1·26	1·32	0·87	0·03	2·22

11-OH E = 11β-Hydroxyaetiocholanolone.
11-OH A = 11β-Hydroxyandrosterone.
11-KE = 11-Ketoaetiocholanolone.
11-KA = 11-Ketoandrosterone.
E = Aetiocholanolone.
A = Androsterone.

TABLE V.—*Comparison of the Mean Pre-operative Excretion of 17-ketosteroid Fractions in Twelve Patients Whose Cancer Subsequently Improved Following Endocrine Surgery and in 10 Patients in Whom There Was No Response*

Response to endocrine surgery	Number of patients	11-oxy 17-ketosteroids		11-deoxy 17-ketosteroids	
		Arithmetic	Logarithmic	Arithmetic	Logarithmic
Cancer regressed	12	0·94	0·864	1·83	1·073
Cancer not improved	10	1·23	1·038	2·28	1·236
Mean difference ± S.E.	—	0·29 ± 0·258	0·174 ± 0·130	0·45 ± 0·617	0·163 ± 0·198
P	—	>0·2	>0·1	>0·4	>0·4

Post-operative estimations.—Estimations of neutral 17-ketosteroid fractions on 24-hour or pooled 3-day collections of urine were made in sixteen patients after adrenalectomy and oophorectomy and on pooled 3-day collections in twenty-two patients after Yttrium-90 implantation of the pituitary. In both groups of patients urine was collected within 12 months of surgery while cortisone therapy (50 mg./day) was being taken. Significant amounts of 11-oxy 17-ketosteroids were present in the urine of all patients, being the excretion products of maintenance cortisone (Fig. 4 and 5). The amounts of these fractions were not related to the clinical response (Tables VII and VIII). The means in the groups of patients were statistically compared as above ; no significant difference was demonstrated (Table IX).

Adrenalectomy and oophorectomy led to the complete disappearance of 11-deoxy 17-ketosteroids in four of ten patients whose cancer responded to the operation and in five of six patients whose cancer did not improve. In six patients in the responding group trace amounts of aetiocholanolone only were detected while in one patient who failed to benefit, small but significant amounts of aetiocholanolone and androsterone were present (Table X).

Implantation of the pituitary with Yttrium-90 was less effective in reducing the excretion of 11-deoxy 17-ketosteroids although in five of twelve patients whose cancer regressed and in four of ten patients who had no response, no measurable amounts of these fractions were present in the urine. In the other patients in both groups variable amounts of one or more fractions were detected (Table XI).

The mean outputs of 11-deoxy 17-ketosteroids were statistically compared in patients who responded and in those who failed to improve following pituitary implantation with Yttrium-90. No significant difference was present (Table XII).

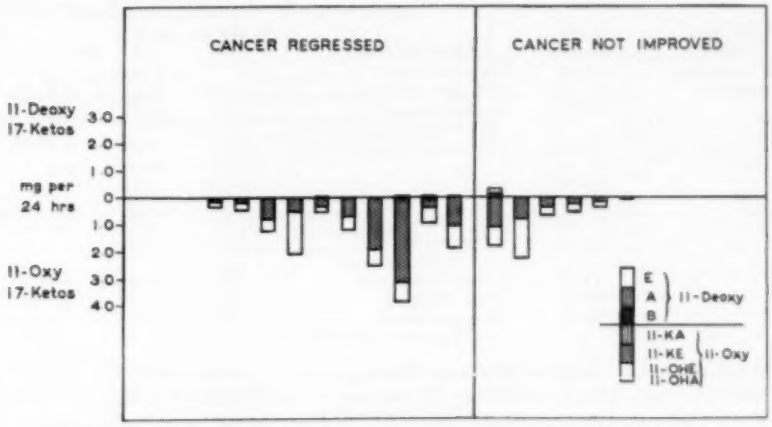

FIG. 4.—Urinary 17-ketosteroid fractions in sixteen women with advanced breast cancer treated by adrenalectomy and oophorectomy.

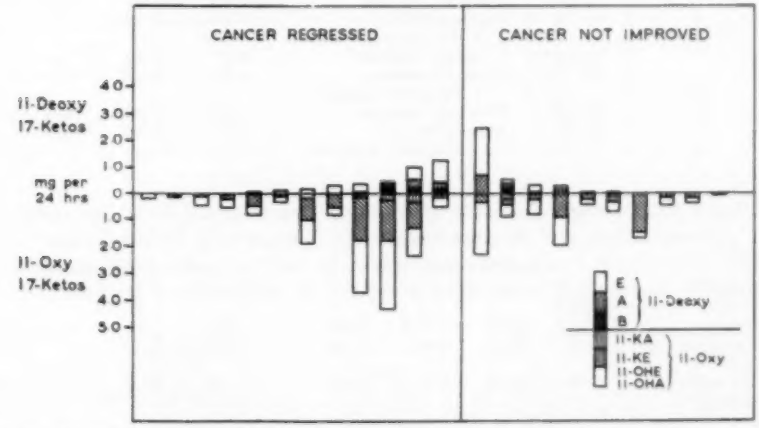

FIG. 5.—Urinary 17-ketosteroid fractions in twenty-two patients with advanced breast cancer treated by implantation of the pituitary with Yttrium-90.

DISCUSSION

The estimation of total neutral 17-ketosteroids in female urine is a crude and relatively non-specific index of adrenocortical activity. In patients with breast cancer their excretion was consistently reduced by adrenalectomy and oophorectomy, irrespective of the clinical response of the patient, a finding previously

TABLE VI.—*Pre-operative Estimations of Urinary 17-ketosteroid Fractions in Patients with Breast Cancer Treated by Endocrine Ablation.*

Behaviour of cancer	Case number	Endocrine Surgery	17-ketosteroid fraction (mg./day) 11-deoxy 17KS	11-oxy 17 KS	Ratio : 1	Mean ratio
Cancer regressed (12 patients)	Ox 22	Oophorectomy	0	0·26	..	
	S 20	Pituitary implant	0·34	0·49	0·7	
	Ys 16	Oophorectomy + Pituitary implant	1·20	1·33	0·9	
	Ys 10	Pituitary implant	1·39	1·15	1·2	
	S 39	" "	0·27	0·19	1·4	
	Ys 13	" "	3·10	2·06	1·5	
	Ys 14	" "	2·33	1·45	1·6	2·2 : 1
	S 44	" "	2·47	0·97	2·5	
	As 6	Adrenalectomy + Oophorectomy	2·63	1·03	2·6	
	Ys 27	Pituitary implant	1·24	0·48	2·6	
	Ox 1	Oophorectomy	5·48	1·62	3·4	
	As 38	Adrenalectomy + Pituitary implant	1·55	0·27	5·7	
Cancer not improved (10 patients)	Ys 25	Pituitary implant	0·26	1·59	0·2	
	S 1	Oophorectomy + Pituitary implant	2·36	2·56	0·9	
	Ys 9	Pituitary implant	1·38	1·00	1·4	
	As 41	Oophorectomy + Adrenalectomy + Pituitary implant	0·44	0·32	1·4	
	Ys 12	Pituitary implant	1·43	0·94	1·5	2·1 : 1
	Ys 11	Oophorectomy + Pituitary implant	3·39	1·56	2·2	
	Ys 18	Pituitary implant	3·07	1·29	2·4	
	As 5	Adrenalectomy + Pituitary implant	2·85	0·98	2·9	
	Ys 15	Pituitary implant	4·29	1·22	3·5	
	Ys 10	Adrenalectomy + Pituitary implant	3·35	0·82	4·1	

TABLE VII.—*Mean Excretion of Urinary 17-ketosteroid Fractions After Adrenalectomy and Oophorectomy in 16 Women with Breast Cancer*

Response to endocrine surgery	11-oxy 17-ketosteroids: 11OH E 11OH A	11 KE	11 KA	Total	11-deoxy 17-ketosteroids: E	A	β	Total
Cancer regressed (10 patients)	0·58	0·92	0·02	1·52	0·01	0	0	0·01
Cancer not improved (6 patients)	0·49	0·46	0	0·95	0·04	0·04	0	0·08

TABLE VIII.—*Mean Urinary Excretion of 17-ketosteroid Fractions After Pituitary Implantation with Yttrium-90 in 22 Women with Breast Cancer*

Response to endocrine surgery	11-oxy 17-ketosteroids: 11OH E 11OH A	11 KE	11 KA	Total	11-deoxy 17-ketosteroids: E	A	β	Total
Cancer regressed (12 patients)	0·73	0·59	0·06	1·38	0·18	0·05	0·06	0·29
Cancer not improved (10 patients)	0·53	0·39	0·04	0·96	0·23	0·10	0·02	0·35

TABLE IX.—*Comparison of Mean Postoperative Excretion of* 11-*oxy* 17-*ketosteroids in* 10 *Patients Who Responded and* 6 *Patients Who Did Not Respond to Adrenalectomy and in* 12 *Patients Who Responded and* 10 *Patients Who did Not Respond to Pituitary Implantation*

Nature of endocrine surgery	Response to endocrine surgery	Number of patients	Mean 11-oxy-17-ketosteroid excretion	
			Arith.	Log.
Oophorectomy and Adrenalectomy	Cancer regressed	10	1·51	1·063
	Cancer not improved	6	0·93	0·770
	Mean difference	—	0·58 ± 0·540	0·293 ± 0·212
	P	—	>0·3	>0·1
Pituitary implantation	Cancer regressed	12	1·38	0·916
	Cancer not improved	10	0·96	0·839
	Mean difference	—	0·419 ± 0·509	0·077 ± 0·191
	P	—	>0·4	>0·6

TABLE X.—*The Excretion of Urinary* 11-*deoxy* 17-*ketosteroids After Adrenalectomy in* 7 *Women With Breast Cancer. In the Other* 9 *Patients Studied After Adrenalectomy and Oophorectomy No* 11-*deoxy* 17-*ketosteroids Were Present*

Response to endocrine surgery	Patient	11-deoxy 17-ketosteroids		
		E	A	β
Cancer regressed	As 17	0·03	0	0
	As 9	0·01	0	0
	As 7	0·04	0	0
	As 38	0·01	0	0
	As 26	0·01	0	0
	Ax 4	0·02	0	0
Cancer not improved	As 5	0·16	0·15	0

TABLE XI.—*The Excretion of Individual* 11-*deoxy* 17-*ketosteroids After Pituitary Implantation With* ^{90}Y *in* 13 *Women With Breast Cancer*

Response to endocrine surgery	Patient	11-deoxy 17-ketosteroids		
		E	A	β
Cancer regressed	Ys 14	0·20	0	0·10
	S 3	0·83	0·19	1·20
	Yu 10	0·26	0	0
	Ys 6	0·45	0·35	0·14
	Ys 3	0·07	0·02	0·27
	Ys 1	0·09	0	0
	Ys 22	0·04	0	0·01
Cancer not improved	Ys 15	0·16	0·11	0
	Ys 10	1·72	0·68	0
	Ys 25	0·28	0	0·01
	S 37	0·02	0·04	0
	S 66	0·12	0·19	0·17
	Ys 23	0	0	0·01

TABLE XII.—*Comparison of Mean Excretion of 11-deoxy 17-ketosteroids in 12 Patients Who Responded and 10 Patients Who did Not Respond to Pituitary Implantation*

Response to endocrine surgery	Number of patients	Mean 11-deoxy-17-ketosteroids	
		Arith.	Log.
Cancer regressed	12	0·27	0·292
Cancer not improved	10	0·35	0·295
Mean difference	—	0·084 ± 0·250	0·003 ± 0·184
P	—	>0·8	>0·9

reported by Strong *et al.* (1956). In the adrenalectomized patient the small amounts of total 17-ketosteroids excreted in the urine are metabolites of maintenance cortisone, and if this is temporarily stopped they fall to undetectable levels (Hobkirk *et al.*, 1959).

In women with breast cancer whose adrenals have not been removed ACTH administration leads to increased excretion of urinary total 17-ketosteroids. This effect was not noted in any of the adrenalectomized patients studied, indicating that these patients were without functionally active adrenocortical tissue even when their cancer failed to respond, a finding which has recently been confirmed by more sensitive hormone assays (Sim *et al.*, unpublished).

Unlike adrenalectomy and oophorectomy, implantation of the pituitary with Yttrium-90 did not reduce total 17-ketosteroid excretion either in responding or non-responding patients. Atrophy of the adrenals after pituitary implantation is a gradual process and persistent adrenal steroidgenesis may account for the higher ketosteroid excretion compared with adrenalectomized patients (Forrest, Sim and Stewart, 1960).

Fractionation of the urinary neutral 17-ketosteroids provides a more sensitive and reliable estimate of adrenocortical function. Our results indicate that the pre-operative levels of these fractions are not of value in forecasting the response to endocrine surgery, a finding in agreement with that of Plantin *et al.*, (1958). Moreover, they do not confirm the recent suggestion by Allen, Hayward and Merivale (1957, 1958), that the ratio of 11-deoxy to 11-oxy 17-ketosteroids in the urine of patients with breast cancer is useful in this respect, although it must be stressed that these workers used methods of estimation different from ours and those of the Stockholm group.

After adrenalectomy and oophorectomy 11-oxy 17-ketosteroids are still present in the urine provided maintenance cortisone is taken, and they are of value in indicating persistent adrenocortical function only if maintenance cortisone is withdrawn (Hobkirk *et al.*, 1959 ; Sim *et al.*, unpublished). After pituitary implantation also, cortisone is required to maintain good health and metabolites will be excreted as 11-oxygenated steroids. It is therefore not surprising that the levels of 11-oxy 17-ketosteroids found in the urine after adrenalectomy and oophorectomy and after destruction of the pituitary bear no relationship to the clinical response.

The 11-deoxy 17-ketosteroids are mainly metabolites of the C19 androgenic steroids and their excretion is unlikely to be influenced by cortisone therapy (Holliday, Kellie and Wade, 1960). Their presence in the urine of women with breast cancer is thus highly suggestive of the presence of functioning adrenocortical, or in the case of aetiocholanolone, possibly ovarian tissue (Plantin *et al.*, 1958).

These steroids were completely absent from the urine of nine of sixteen patients after adrenalectomy and oophorectomy. In six of the remaining seven patients small amounts of aetiocholanolone (0·01–0·04 mg./24 hr.) only were detected and in only one patient whose cancer did not improve following adrenalectomy, were both aetiocholanolone (0·16 mg./24 hr.) and androsterone (0·15 mg./24 hr.) found. This latter patient did not have any clinical response to subsequent pituitary implantation with Yttrium-90 (100 per cent destruction) and no adrenal rests were found post mortem.

Other workers have also described small amounts of aetiocholanolone in the urine of adrenalectomized-oophorectomized patients (Kellie, 1954; Kellie and Wade, 1957; Bulbrook, Greenwood and Thomas, 1958). With the method we used the quantitative significance of amounts of less than 100 μg. is doubtful although they can be quantitatively detected down to a level approximating 10 μg.; it is quite clear that the presence of such small amounts of this steroid in the urine of adrenalectomized patients does not preclude benefit from the operation.

Implantation of the pituitary with Yttrium-90 was less effective in reducing the excretion of these fractions and aetiocholanolone (0·02–1·72 mg./24 hr.) was found in the urine of twelve and androsterone (0·02–0·68 mg./24 hr.) and β fraction (0·01–1·20 mg./24 hr) in the urine of seven of the twenty-two patients studied. The amounts of these steroids in the urine were not related to the clinical response.

Aetiocholanolone and androsterone have also been recovered from the urine of women with breast cancer after surgical hypophysectomy (Holliday, Kellie and Wade, 1958) although these workers could not isolate dehydroepiandrosterone from the urine of any of the twelve patients studied.

In our experience 11-deoxy 17-ketosteroids may still be recovered from the urine when the intrasellar pituitary is completely destroyed, although the levels are generally low provided 90 to 100 per cent of the gland is necrosed (Forrest *et al.*, 1960). It is known that aldosterone secretion persists despite complete hypophysectomy (McLean *et al.*, 1957) and evidence has also been presented that cortisol synthesis also may continue in the absence of a functioning pituitary gland (Forrest *et al.*, 1960). It does not seem unreasonable to suppose that the autonomous adrenal may also be able to synthesize C19 steroids, albeit in reduced amount, and further evidence of this has recently been reported by Wilson, Lipsett and Butler, (1960).

The presence of 11-deoxy 17-ketosteroids in the urine of women whose breast cancer had regressed following pituitary implantation clearly indicates that pituitary destruction can produce a beneficial response in the absence of full adrenal suppression. This would suggest that deprivation of a pituitary hormone other than corticotrophin was primarily responsible for its effect on tumour growth.

SUMMARY

Urinary 17-ketosteroids and their fractions have been estimated in women with breast cancer before and after adrenalectomy and oophorectomy and implantation of the pituitary gland with Yttrium-90. No relationship has been found between pre-operative levels and the response to endocrine surgery. Following adrenalectomy and oophorectomy 17-ketosteroids with an 11-oxygen function, which are derived from maintenance cortisone, persist in the urine whereas those without

this function are generally absent. Trace amounts of aetiocholanolone may still be found in the urine of some patients after adrenalectomy and oophorectomy but this is not of significance in relation to their response to the operation.

Following pituitary implantation with Yttrium-90 both 11-oxy and 11-deoxy 17-ketosteroids are more consistently found in the urine indicating that full adrenal suppression is not required for benefit from this operation.

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PLASMA β-GLUCURONIDASE LEVELS IN BREAST CANCER

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IT has been established by Kerr and Levvy (1947) and Levvy, Kerr and Campbell (1948) that there is a connection between the level of tissue β-glucuronidase and processes of growth and repair.

Fishman, Kasdon, Bonner, Fishman and Homburger (1951) have shown that alterations in the hormonal state of the subject may have a profound effect on the level of the enzyme in the blood and tissues.

The blood level of β-glucuronidase in 23 patients with breast cancer has been investigated by Cohen and Huseby (1951) who found the difference between normal controls and cancerous subjects to be barely significant.

Goldbarg, Pineda, Banks and Rutenberg (1959) however found raised blood levels in 14 out of 21 cases of breast cancer especially in those with liver secondaries.

Boyland, Gasson and Williams (1957) observed high β-glucuronidase levels in the urine of patients with malignant disease, including carcinoma of the breast.

The present work deals with the plasma β-glucuronidase levels in a series of 47 patients suffering from malignant disease of the breast and also with factors which may have influenced these levels.

METHOD

Blood was taken into oxalate bottles. the plasma separated as soon as possible and stored in rubber stoppered glass tubes at −20° C. for periods of 1–5 days. In order to minimize diurnal variation the specimens were collected whenever possible at the same time of day, namely 1.30 p.m.

The loss of activity of the enzyme due to storage was of the order of 10 per cent over 7 days.

The method of estimation has been that of Tallalay, Fishman and Huggins (1946) as modified by Boyland, Wallace and Williams (1955) and Boyland, Gasson and Williams (1957). The substrate, phenolphthalein mono-β-glucuronic acid (0·05 g. Sigma) was dissolved in 20 ml. ethanol and diluted to 100 ml. with water. The plasma specimens were diluted 1 in 10 with water immediately before incubation. Diluted plasma (1 ml.), substrate solution (1 ml.) and acetate buffer (1 ml., 0·1 M pH 4·5) were incubated in duplicate stoppered tubes at 37° C. for 16 hours. A blank containing no plasma was incubated. At the end of incubation 1 ml. of 10 per cent Na_2CO_3 solution was added to each tube and 1 ml. of diluted plasma to the blank. The tubes were centrifuged for ten minutes at 3000 r.p.m. and read against the blank on a Unicam S.P. 500 spectrophotometer at 550mμ. The activity was expressed in units, 1 unit liberating1 μg. of phenolphthalein per ml. of plasma per hour at 37° C.

MATERIAL

Fifty normal women and 47 patients suffering from breast carcinoma were studied. A total of 368 estimations was performed on these subjects both before and after treatment. One hundred and ninety-three of these estimations were performed before the patients received treatment (apart from androgens which in many cases had been given before the patient was referred, and operative procedures such as removal of the primary lesion which in some cases had been performed many years before) and these 193 estimations form the basis of this report. It is hoped to publish the findings with regard to the effect of treatment on the enzyme levels in a later report.

1. *Normal controls.*—Fifty normal women aged from 18 to 70 years served as controls (Table I). Thirty-four of these were hospital patients about to undergo operation for hernias, varicose veins, etc. The remainder were students and members of the hospital staff. Thirty of the 50 were premenopausal and 20 menopausal or postmenopausal. The overall mean value of β-glucuronidase for these 50 individuals was 3·58 units and the standard deviation 1·31. The normal range was taken as $\pm 2\sigma$ from the mean, i.e. 0·96 to 6·20 units.

The mean values for the pre- and postmenopausal groups were almost identical being 3·57 and 3·59 respectively.

2. *Cases of breast cancer.*—The enzyme levels and relevant clinical details for the 47 cases of breast cancer are summarized in Table II. The mean β-glucuronidase for this group irrespective of staging or previous therapy is 6·90 units (standard deviation 3·87). This figure is significantly greater than the mean value of the control group ($c = 5{\cdot}63$ $P = < 0{\cdot}0000001$). Fifty-one per cent of these cases had enzyme levels within the normal range and 49 per cent had elevated readings.

Analysis of cases

An analysis of these figures was undertaken in an attempt to correlate them with the clinical and pathological findings in individual cases. The results are expressed graphically in Fig. 1.

(a) *Clinical staging.*—Of the 47 cases 10 (21·25 per cent) were clinically classed as stage I or II (Table II). The mean value for this group is 6·13 units (standard deviation 4·07). A comparison of this with the control group shows the difference to be significant ($t = 3{\cdot}67$ $P = < 0{\cdot}001$).

The remaining 37 cases were classed as stage III or IV and the mean value for this group is 7·11 (standard deviation 3·84). Comparison with the control group gives a value for c of 5·35 and for P of $< 0{\cdot}0000001$.

Comparison of the stage III and IV group with the stage I and II group however shows a difference in means of only 0·98. This does not attain statistical significance ($t = 0{\cdot}71$ $P = < 0{\cdot}5$).

(b) *Previous hormone therapy.*—Of the 37 cases in the stage III and IV group 20 had received treatment with androgenic hormones usually methyl testosterone by mouth or testosterone propionate by injection at some time in the course of their disease.

Cases 31, 33, 34, and 38 in the androgen treated group and 32, 35, 36, and 37 in the non-treated group had clinical evidence of liver involvement at the time of the estimations and are excluded. This leaves for comparison 16 treated and 13 untreated cases. The mean values of β-glucuronidase for these two groups

are 7·99 and 5·61 units respectively. With the number of cases available this does not indicate a significant difference ($t = 1·56$, $P = < 0·2$).

(c) *Hepatic involvement.*—Twenty-nine of the 37 cases in the stage III and IV group had no clinical or laboratory evidence of liver damage. Nine cases had either enlarged livers, jaundice or abnormal liver function tests. In one case (No. 26) observations were made both before and after the development of signs of liver metastasis and she is therefore included in both groups.

TABLE I.—*Normal Controls*

Control number	Age	Relation to menopause	Number of readings	Mean β-glucuronidase
1	30	Premenopausal	1	4·30
2	37	,,	1	1·85
3	22	,,	1	1·27
4	18	,,	3	1·40
5	21	,,	1	3·13
6	22	,,	1	2·25
7	28	,,	1	2·48
8	25	,,	1	3·24
9	32	,,	1	3·40
10	32	,,	1	3·16
11	27	,,	1	3·51
12	22	,,	1	2·75
13	39	,,	1	5·01
14	31	,,	1	3·86
15	62	Postmenopausal	1	4·53
16	63	,,	1	2·75
17	44	Premenopausal	1	2·32
18	55	Postmenopausal	1	1·97
19	52	Menopausal	2	4·87
20	55	Postmenopausal	1	4·60
21	53	Menopausal	1	2·35
22	63	Postmenopausal	1	3·80
23	65	,,	1	3·36
24	40	Premenopausal	1	1·67
25	70	Postmenopausal	1	4·95
26	21	Premenopausal	1	5·00
27	55	Menopausal	1	2·44
28	21	Premenopausal	1	3·95
29	21	,,	1	3·66
30	21	,,	1	3·86
31	18	,,	1	3·90
32	62	Postmenopausal	1	3·90
33	52	Menopausal	1	2·32
34	28	Premenopausal	1	4·60
35	54	Postmenopausal	1	6·20
36	57	,,	1	3·20
37	29	Premenopausal	1	4·50
38	39	,,	1	8·50
39	28	,,	1	4·85
40	22	,,	1	4·20
41	40	,,	1	2·44
42	57	Postmenopausal	1	5·05
43	51	,,	1	4·10
44	49	,,	1	2·73
45	37	Premenopausal	1	4·00
46	46	Postmenopausal	1	3·86
47	39	Premenopausal	1	3·30
48	61	Postmenopausal	1	2·00
49	29	Premenopausal	1	4·61
50	46	Postmenopausal	1	2·84

The mean β-glucuronidase level of the group without liver secondaries was 6·93 and of the other group 7·26. This difference is not significant ($t = 0·34$, $P = < 0·8$). Five of the cases in the hepatic group were actually jaundiced at one stage of their disease. The mean value for this group (No. 26, 32, 34, 37 and

TABLE II.—*Breast Cancer*

Case number	Age	Relation to menopause	Androgen therapy	Hepatic metastasis	Histology	Number of readings	Mean β-glucuronidase	
1	80	Post.	—	—	Spheroidal	1	10·47	
2	48	Men.	—	—	,,	1	14·70	
3	55	Post.	—	—	,,	1	4·85	
4	71	,,	—	—	Poorly diff.	1	5·91	Stage I
5	68		—	—	,, ,,	1	3·70	
6	59	,,	—	—	Mucoid.	1	2·42	
7	71	,,	—	—	Scirrhous	1	1·85	
8	48	,,	—	—	Spheroidal	1	7·70	
9	34	Pre.	—	—	,,	1	2·43	Stage II
10	34	,,	—	—	Anaplastic	1	7·25	
11	47	,,	—	—	Not known	4	2·55	
12	49	,,	—	—	Undiff.	3	1·90	
13	55	Post.	—	—	Spheroidal	2	3·50	
14	76	,,	+	—	,,	8	5·87	
15	51	,,	+	—	Scirrhous	3	4·84	
16	54	,,	—	—	Not known	4	18·62	
17	35	Pre.	+	—	Undiff.	4	18·80	
18	52	Men.	+	—	Scirrhous	1	5·05	
19	42	Pre.	+	—	Anaplastic	1	9·00	
20	46	Rad. men.	+	—	,,	6	7·32	
21	79	Post.	—	—	,,	2	4·12	
22	37	Rad. men.	+	—	,,	2	11·50	
23	78	Post.	—	—	Not known	10	7·52	
24	58	,,	—	—	Scirrhous	2	5·27	
25	73	,,	—	—	,,	8	5·79	
26	32	Pre.	+	—	,,	5	4·32	
27	56	Men.	+	—	Spheroidal	4	3·67	
28	65	Post.	+	—	Not known	1	10·25	
29	49	Pre.	+	—	Spheroidal	3	11·25	
30	54	Post.	+	—	Anaplastic	3	4·05	Stages III and IV
31	47	Pre.	+	+	Not known	5	6·58	
32	48	Post.	—	+	,, ,,	2	4·73	
33	54	,,	+	+	Scirrhous	10	7·99	
34	52	,,	+	+	Not known	1	6·60	
35	55	,,	—	+	Scirrhous	2	4·72	
36	58	,,	—	+	Paget's and scir.	5	8·83	
37	53	,,	—	+	Not known	3	12·90	
38	43	Post-hypo.	+	+	Anaplastic	5	9·85	
39	47	Pre.	—	—	Scirrhous	4	4·36	
40	65	Post.	—	—	Not known	3	4·57	
41	58	,,	—	—	Spheroidal scirrhous	1	4·33	
42	52	,,	+	—	Mucoid	2	8·80	
43	59	,,	+	—	Not known	6	9·44	
44	45	,	+	—	,, ,,	1	7·00	
45	63	,,	+	—	,, ,,	6	6·66	
46	62	,,	—	—	,, ,,	1	4·00	
47	54	,,	—	—	Scirrhous simplex	2	6·35	
26b	..	..	+	+	..	2	6·62	

38) during the jaundiced period was 8·59 which of course, for so few cases is not outside the range of chance deviation.

(d) *Histology.*—The histology of the lesion was known in 34 of the 47 cases. Twenty-one of these were described as anaplastic and the remainder were scirrhous carcinomas of varying degrees of differentiation. The mean values of glucuronidase for the two groups are 7·41 and 5·16 ($t = 1·76$, $P = < 0·1$).

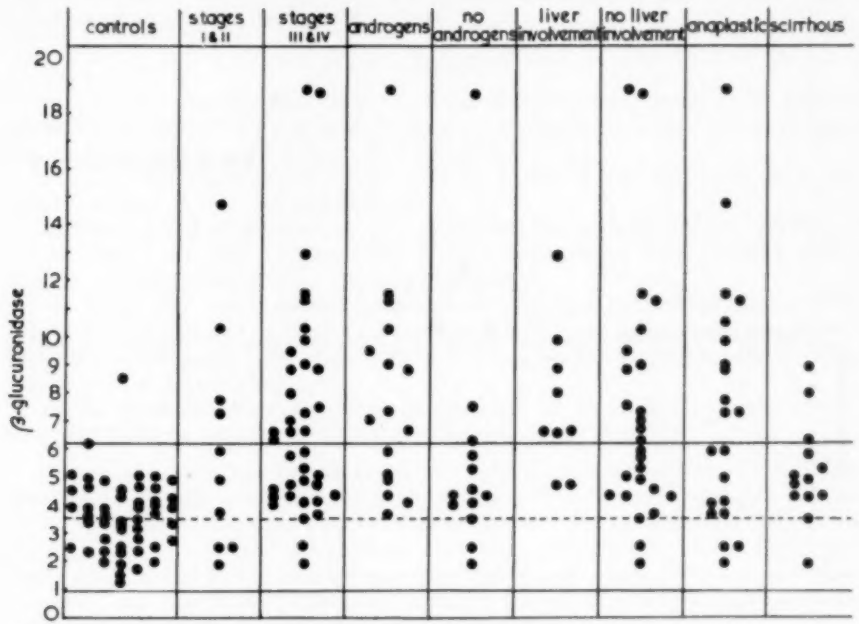

FIG. 1.—Comparison of plasma β-glucuronidase levels of 50 normal women with 47 cases of breast cancer. The dotted horizontal line represents the mean value for the control group and the upper and lower continuous lines two standard deviations around this mean.

DISCUSSION

The difference in levels between cancerous and non-cancerous individuals in this series is highly significant. This corresponds with the findings of Goldbarg, Pineda, Banks and Rutenburg (1959), but not with those of Cohen and Huseby (1951).

The reason for this discrepancy between different workers is not clear. The method used by Cohen and Huseby (1951) is that of Tallalay, Fishman and Huggins (1946) while Goldbarg, Pineda, Banks and Rutenburg (1959) used 6-bromo-2-naphthyl β-D-glucopyruronoside as a substrate.

The method used in the present series is a modification of the method of Tallalay, Fishman and Huggins (1946) suggested by Boyland, Wallace and Williams (1955).

The estimations have been carried out on oxalated plasma without the addition of protein precipitating agents such as the trichloracetic acid advocated by Fish-

man, Springer and Brunetti (1948) and also used by Goldbarg, Pineda, Banks and Rutenburg (1959).

The difference in means between the stage I and II group and the stage III and IV group is not sufficiently large in relation to the number of cases to be outside the range of chance but serial observations on individual patients have frequently demonstrated a rising titre corresponding to deterioration in clinical state. Such a case is illustrated in Fig. 2.

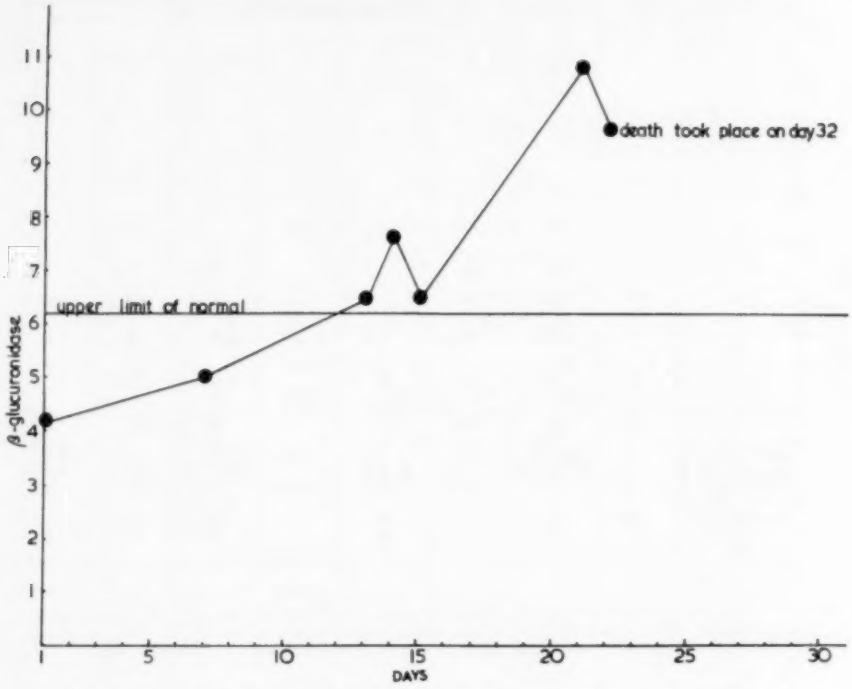

FIG. 2.—Serial estimations of plasma β-glucuronidase (case 23) showing rise in levels with advance in disease process.

It seems likely that with larger numbers of cases this gradient will attain significant proportions.

The findings with regard to hepatic metastasis do not correspond with those of Goldbarg, Pineda, Banks and Rutenburg (1959) who found raised levels in a majority of cases of hepatic metastasis from breast cancer. Though in the 5 jaundiced cases very high levels were seen which suggest again that with a larger number of cases the difference might be significant.

The correlation with histological grading is of interest, suggesting as it does, the possibility that there may be less β-glucuronidase activity in those patients with scirrhous types of growth.

The question of hormonal effects on tumours and on β-glucuronidase is of

considerable complexity and these results do not as yet allow of any but the most tentative suggestions as to their relationship. It is perhaps of interest, however, to note that the ratio of normal to abnormal levels in the cancer group (51 and 49 per cent) is not very far removed from the percentages given by Baron, Gurling and Radley-Smith (1958) and Luft, Olivecrona, Ikkos, Nilsson and Ljunggren (1956) for remissions and failures following hypophysectomy. This may of course be entirely coincidental but investigations are in progress to ascertain whether any relationship exists.

SUMMARY

(1) An investigation into the plasma β-glucuronidase levels of 47 cases of breast cancer and 50 normal women has been undertaken using a modification of the method of Tallalay, Fishman and Huggins (1946).

(2) The difference between the enzyme levels of the cancerous and non-cancerous groups was highly significant.

(3) Stage I and II carcinomas were associated with significantly raised enzyme levels. Stage III and IV cases had higher mean values than Stage I and II but the difference was within the range of chance.

(4) Cases who had received androgen therapy had somewhat higher mean values than those who had not had androgens and those with anaplastic growths had higher values than those with scirrhous but chance variation cannot be excluded as a cause for this.

(5) No great difference in enzyme levels could be demonstrated between cases with and without hepatic metastasis in the absence of jaundice.

I wish to express my thanks to Mr. E. J. Radley-Smith and Dr. D. N. Baron for sponsoring this work and for their advice and encouragement; also to Dr. D. C. Williams whose very considerable experience in this field has been of the greatest help in overcoming technical and other difficulties; to the Consultant Staff of the Royal Free Hospital who have generously allowed me access to their patients; and to Sister Hitchcock without whose help and co-operation the collections of specimens would have been impossible.

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THE HISTOGENESIS OF MALIGNANT TUMOURS INDUCED BY COBALT IN THE RAT

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THE production of malignant tumours in rats injected intramuscularly with pure cobalt metal powder has been described already (Heath, 1956). Tumours occurred at the injection site in 17 out of 30 rats over a period ranging from 5 to 12 months after the injection. Thirteen out of the 17 tumours contained a malignant component derived from muscle; malignant connective tissue elements were also present in some of these 13, and in the remaining 4 tumours the malignant process appeared to have arisen predominantly in the connective tissue. The tumours were rhabdomyosarcomata, rhabdomyofibrosarcomata, pleomorphic and fibrosarcomata.

The purpose of the present investigation was to follow step by step and in some detail the tissue changes caused by the injection of powdered cobalt metal into rat skeletal muscle, up to the stage at which frankly malignant changes could be recognised.

MATERIALS AND METHODS

Two experiments were made. In the first the rats were killed at fortnightly intervals; since the results showed that pronounced changes were already present in the tissue at 2 weeks, a second experiment was undertaken in which the animals were killed at intervals of 1 to 28 days after injection.

Male rats of the hooded strain aged 2–3 months were used. Thirty animals were injected in the thigh muscle of the right leg with 0·028 g. cobalt metal powder (spectrographically pure) shaken into suspension with 0·4 ml. of fowl serum; 15 controls were similarly injected with 0·4 ml. fowl serum only. At intervals after the injection animals taken at random were killed by cervical dislocation, and the portion of the thigh muscle surrounding the injection site was excised. Pieces of the excised muscle were fixed in Carnoy's fluid for subsequent staining with methyl green-pyronin (MGP), and in Zenker's fluid for Azan staining; tissue fixed in Carnoy's fluid, however, also gave satisfactory results with Azan.

RESULTS

As in the previous experiments, little or no local reaction to the injection could be detected by examination of the intact animal, and there were no general toxic effects. Study of the histological material, however, revealed a distinct and characteristic chain of events in the cobalt-treated rats but nothing of consequence in the controls injected with serum only. The progressive changes in the cobalt-treated animals led from the initial local trauma, through a stage of cell proliferation up to the malignant change and the final development of tumours of the type previously described (Heath, 1956).

There appear to be two main processes at work :—

(1) The response of the muscle to the mechanical injury produced by the injection of the metal grains, as shown by an attempt at regeneration and repair.

(2) The modification of the regenerative and repair process by the chemical action of the cobalt presumably either by slow solution in the tissue fluids to give cobalt ions, or by direct catalysis at the surface of the metal grains.

These two processes are clearly seen in the histological material and are very similar in all rats affected. In a few animals the injury heals normally, and in others which are not considered in detail here, the progressive changes leading to malignancy occur in the connective tissue alone.

The first response of the muscle to the injection appears at 1 day as an infiltration of leucocytes into the spaces between the muscle bundles and fibres, in regions near the primary injury (Fig. 1). This infiltration is still seen at 4 days but by this time many fibroblasts have appeared in the region. Large aggregates of cobalt grains can be found in immediate contact with intact muscle which sometimes shows no evidence of damage (Fig. 2), whereas at sites distant from the cobalt some muscle fibres contain a greatly increased population of nuclei (Fig. 3). A nucleosis of this type has been described by Altschul (1947) who attributes it to the loss of equilibrium in pressure between the nuclei and the sarcoplasm and fibrillae consequent upon the injury. In other damaged muscle fibres both nuclei and striations have disappeared to leave a homogeneous hyaline material.

The muscle continues to degenerate and at 7 days necrotic unstriated bundles often lie between bundles of normal striated fibres (Fig. 4) ; a further breakdown of this hyaline substance into amorphous granular residues now begins. The zones between adjacent muscle fibres, particularly where one fibre is necrotic, become filled with cells of many types, leucocytes, fibroblasts and characteristic fusiform cells. These last (Fig. 5) are sometimes mononucleate and sometimes have 2–5 nuclei ; their cytoplasm is very basophilic and stains deeply with pyronin. They appear to be myoblasts, possibly derived from the injured muscle fibres by the process of " dissociative degeneration " described by previous authors (Pfühl 1937, Betz 1951, quoted by Godman 1957) ; this process is said to involve the freeing of nuclei from a muscle fibre, each with its own complement of endoplasm, to form mononucleate myoblasts. In other areas there are multinucleate cell tubes (Fig. 6) the cytoplasm of which scarcely stains with pyronin, which correspond to the cell tubes of Waldeyer (Waldeyer 1865, quoted by Godman 1957) and represent collapsed muscle fibres. Regeneration has thus begun.

Extensive areas of degeneration are still present at 12 days with increasing amounts of the amorphous granular material, but regeneration is now well under way ; in some zones many long multinucleate basophilic or striated muscle straps are seen (Fig. 7) which sometimes at least are continuous with damaged muscle bundles. Waldeyer's tubes with faintly staining cytoplasm are still present. Collagen fibres have now appeared in some rats and numerous mast cells are scattered amongst them.

So far the picture of the damage due to the injury and of the subsequent attempts at repair and regeneration agree very closely with the findings of Godman (1957) in the repair of infarcted muscle ; the production of fusiform myoblasts, probably from damaged muscle fibres by " dissociative degeneration ", is common in Godman's material as in ours. Between 2 and 3 weeks after the injection of cobalt, however, the tissue response begins to differ from that described by

Godman. Fusiform myoblasts (Fig. 8) continue to be formed but they show progressively less tendency to associate and mature into differentiated fibres. Whereas in the muscle infarction undifferentiated myoblastic elements diminish in number after 16 days, in the cobalt-treated material they continue to increase.

At 4 weeks changes due to the action of the cobalt have spread further from the metal deposit to more distant regions of the muscle (Fig. 9–15). The cobalt granules which are still present are usually surrounded by a narrow band of necrotic material containing pycnotic nuclei and also some degenerate muscle (Fig. 9). Two or three cell widths away there is a broad zone of viable cells of various types but mainly leucocytes and fibroblasts (Fig. 10) beyond which are viable muscle fibres (Fig. 11). At this stage the changes in the muscle fibres are much more readily followed than in the earlier material and the histological

EXPLANATION OF PLATES

FIG. 1.—Infiltration of leucocytes into the space between muscle bundles and fibres. One day after injection of cobalt. Stained Methyl-Green and Pyronin (MGP). ×450.

FIG. 2.—Grains of cobalt metal in intimate contact with apparently undamaged muscle. Four days. MGP. ×450.

FIG. 3.—Increased numbers of nuclei at a site distant from the injected cobalt but in the same muscle. Four days. MGP. ×450.

FIG. 4.—Damaged muscle fibre showing its hyaline, almost homogeneous nature, in contrast with the neighbouring undamaged fibre. Seven days. MGP. ×450.

FIG. 5.—Fusiform cell with very basophilic cytoplasm staining deeply with pyronin and having possibly 5 nuclei. Seven days. MGP. ×450.

FIG. 6.—Multinucleate cell tubes in which the cytoplasm scarcely stains at all with pyronin; these are the cell tubes of Waldeyer. Seven days. MGP. ×450.

FIG. 7.—Region of regeneration containing numerous multinucleate cells of both types, some with cross striations. Twelve days. MGP. ×200.

FIG. 8.—Free myoblast, possibly binucleate, with cytoplasm staining deeply with pyronin. Nineteen days. MGP. ×450.

FIG. 9.—Cobalt granules still present surrounded by a narrow band of necrotic material containing pycnotic nuclei and also some granular degenerate muscle substance. Four weeks. MGP. ×950.

FIG. 10.—Very cellular zone of considerable width lying between cobalt grains and intact muscle fibres. Four weeks. MGP. ×950.

FIG. 11.—Abutment of the cellular zone upon the intact muscle fibres. Four weeks. MGP. ×950.

FIG. 12.—Abnormal fibres with strings of peripherally situated nuclei. Four weeks. MGP. ×950.

FIG. 13.—Damaged muscle fibre with a collection of peripheral nuclei wrinkling up the sarcolemma. Four weeks. Azan. ×950.

FIG. 14.—Damaged muscle fibre with a chain of nuclei disturbing the regular pattern of striation. The nuclei and nucleoli are larger than normal and stain much more deeply. Four weeks. Azan. ×950.

FIG. 15.—Nuclei in affected muscle showing polar caps of pyronin staining endoplasm. Four weeks. MGP. ×950.

FIG. 16.—Two myoblasts in a region containing much collagen. Note the densely staining nucleoli. Six weeks. MGP. ×950.

FIG. 17.—Binucleate myoblasts, and a mitosis. Ten weeks. MGP. ×950.

FIG. 18.—Giant cell similar to those found in the established cobalt-induced tumours. Fourteen weeks. MGP. ×950.

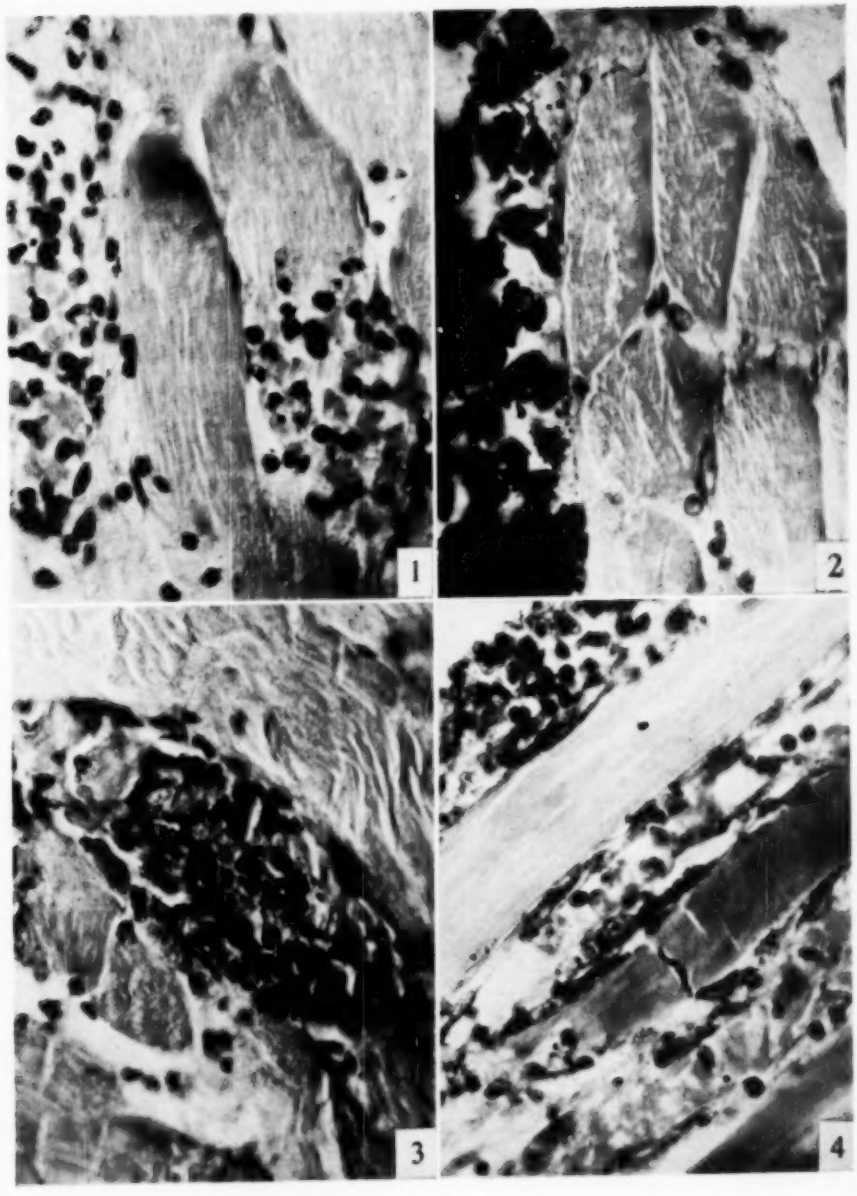
FIG. 19.—Two myoblasts showing cross striations. Fourteen weeks. MGP. ×950.

FIG. 20.—Attempt at production of differentiated muscle fibre in the transplanted cobalt-induced tumour (32nd passage). MGP. ×450.

FIG. 21.—Mitosis in a region with much necrotic material in an early tumour nodule. Twenty weeks. MGP. ×950.

FIG. 22.—Degenerating muscle substance: granular pigmented deposits staining from reddish-brown to yellow and green. Sixteen weeks. MGP. ×950.

FIG. 23.—Mitosis in giant cells in the same nodule as shown in Fig. 21. Twenty weeks. MGP. ×950.



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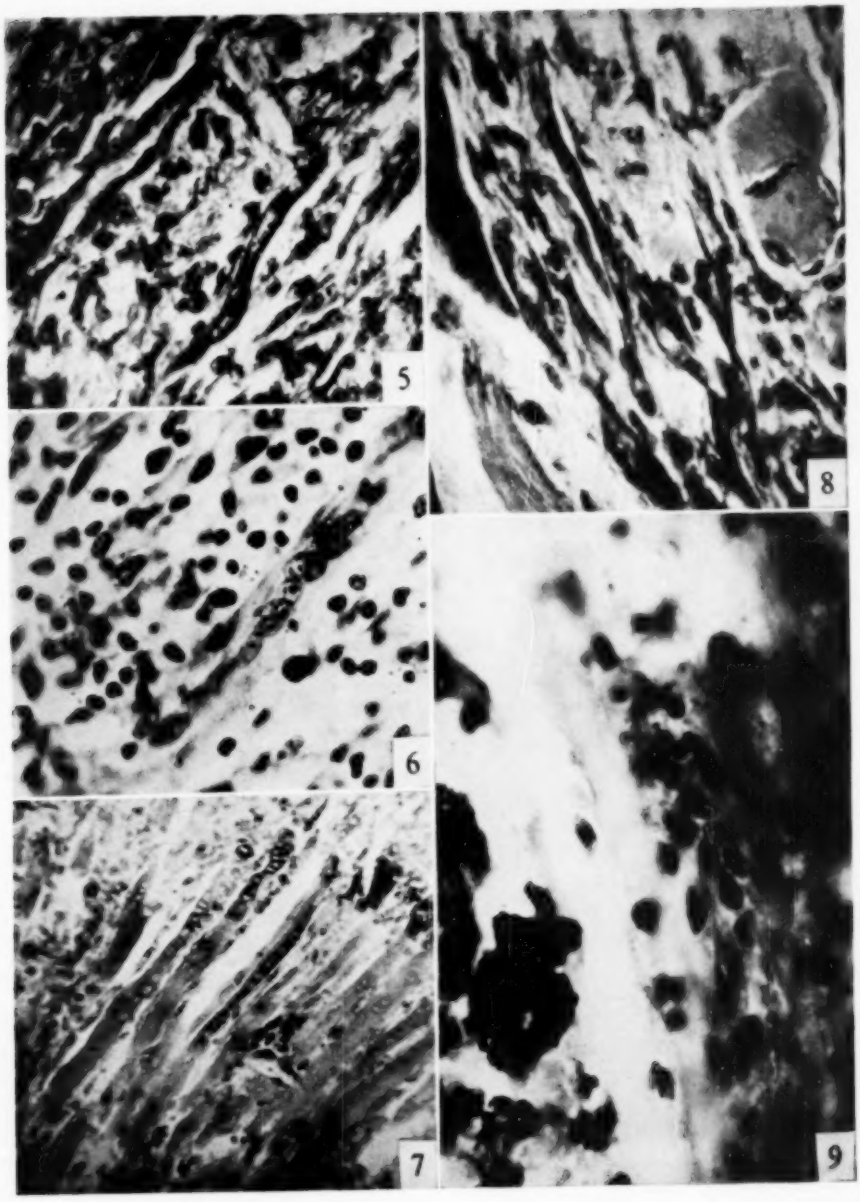

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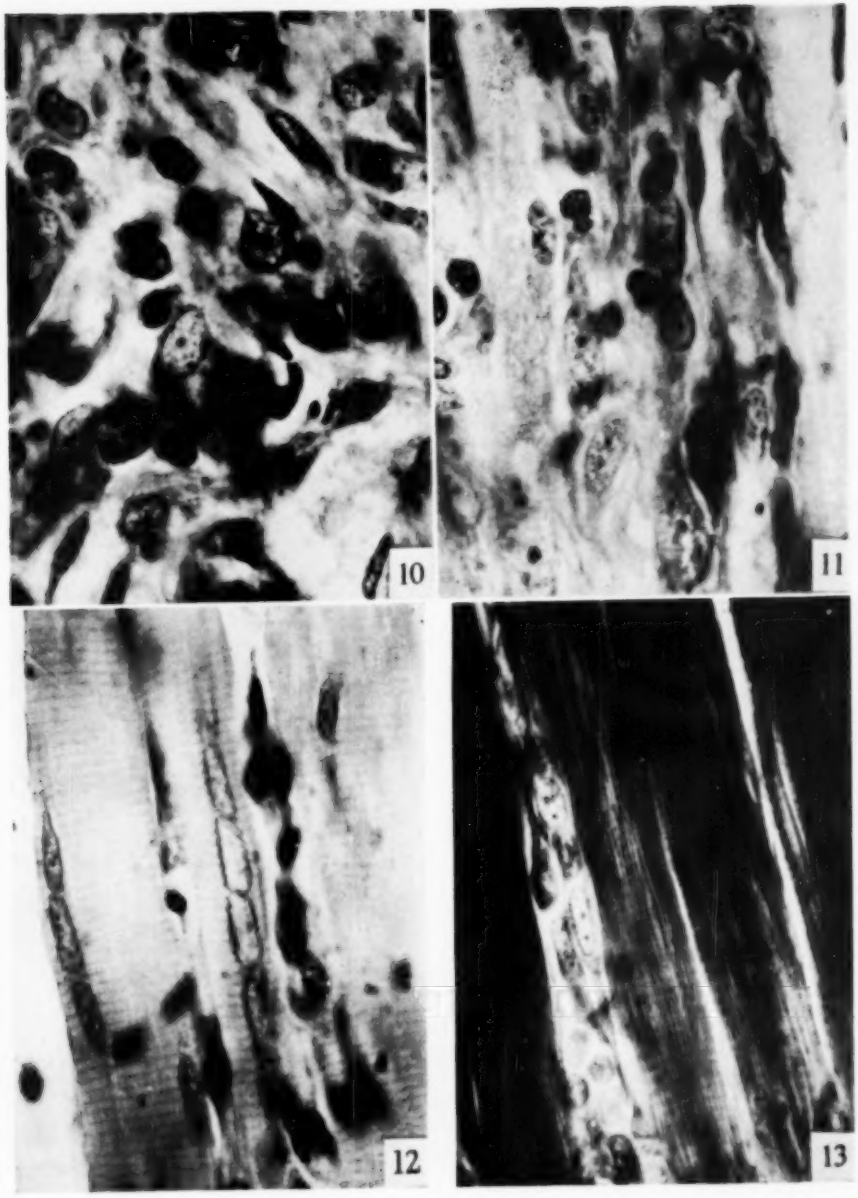
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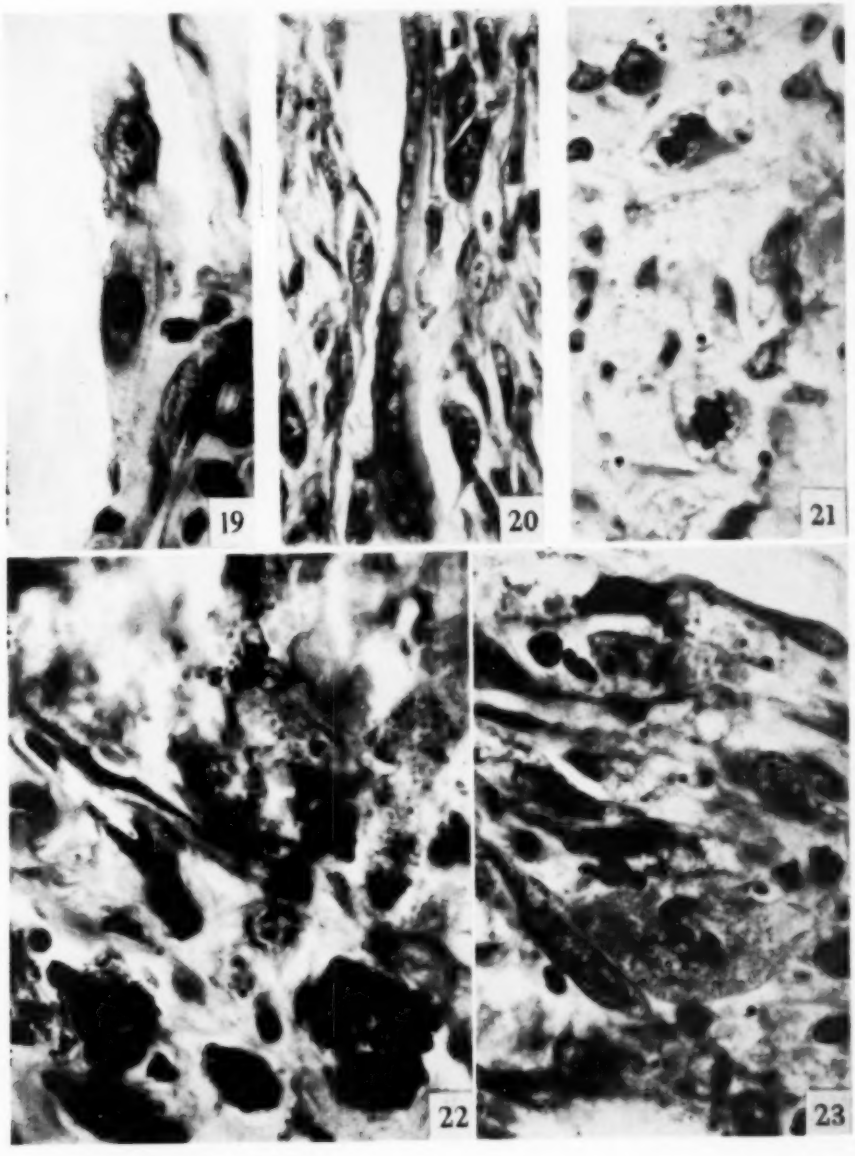

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picture lends support to the view that the mononucleate myoblasts are derived from mature fibres by the dissociative degeneration mentioned above. In the cobalt-treated material muscle fibres closest to the cobalt implant differ from normal fibres in the presence of strings of nuclei along the periphery (Fig. 12); the nuclei may be so close to the surface of the cytoplasm that the sarcolemma is wrinkled and the striations disturbed giving the appearance of an outpouring of nuclei from the interior of the muscle fibre (Fig. 13 and 14). In some areas both the nuclei and nucleoli in these strings are larger and more deeply staining than normal (Fig. 14); other nuclei are associated with large polar caps of basophilic pyronin-staining material (Fig. 15) the so-called endoplasm referred to above, which according to Altschu[illegible]-7) is the true cytoplasm of the muscle cells and independent of the sarcopl[illegible] and fibrillae of the muscle-fibre proper. This "dedifferentiation" of mature muscle fibres continues under the action of cobalt whereas an isolated mechanical trauma only evokes a limited degree of dissociation sufficient to repair the damage (Godman, 1957). Free myoblasts appear in the zones near the cobalt injection; at 6 weeks these cells are larger than in the initial stages of the tissue response, assume more and more abnormal forms, and usually have very basophilic cytoplasm and large deeply staining nucleoli. They may be present between existing muscle bundles, in the residual necrotic material, and in masses of collagen (Fig. 16).

At 8 to 10 weeks free myoblasts are very abundant and many are binucleate. Mitoses are now seen but whether in myoblasts or other cell types has not been established (Fig. 17). At 14 weeks giant cells (Fig. 18) appear in increasing numbers and although their origin is not certain, similar cells are common in the fully developed cobalt-induced tumour. Myoblasts abound, and while some show cross-striations (Fig. 19) most do not.

The continuing action of cobalt on the newly formed myoblasts probably prevents them from redifferentiating into striated elements as they do in repair after infarction. That some capacity for redifferentiation may persist at least in some cells is suggested by the fact that multinucleate straps occasionally appear even in cobalt-induced tumours that have been transplanted for many generations (Fig. 20).

At 16 weeks muscle substance continues to degenerate into pigmented granular masses (Fig. 22) and mitoses are now very frequent, in spite of the fact that cobalt is still present; they even occur in the largely necrotic regions adjacent to metal grains. In one rat cobalt is still present at 20 weeks and a tumour nodule is just discernible (Fig. 21, 23).

DISCUSSION

The study of the histogenesis of these cobalt-induced tumours has revealed that under the experimental conditions described, cobalt causes an extensive and continuing reversal of the normal processes whereby embryonic mononucleate myoblasts associate to form fully differentiated muscle fibres (Firket, 1957). It seems clear that it is from myoblasts of this type, probably originating through the breakdown of mature fibres, that the malignant variants arise and that these in turn develop into the established tumour.

The process is of interest partly because the breakdown of such a highly differentiated tissue is very easy to follow visually, and partly because the production of such tumours from muscle by experimental carcinogens is either uncommon or

has not been often reported. The fact that the carcinogen in these studies is an element, the metal cobalt, and therefore can be followed throughout the organism by various chemical and tracer techniques will enable the investigation to be pursued further in several directions, and gives some grounds for hope that the nature of one particular carcinogenic mechanism can be found.

The work of several authors has shown that cobalt can inhibit the respiration of various tissue and cells. Unpublished experiments by my colleagues, Dr. M. Webb, Mr. J. T. Dingle, Dr. M. R. Daniel and myself, now in progress in this Laboratory, show that cobalt is a poison for some respiratory systems of rat tissues. Experiments are being made to find whether there is a correlation between the dedifferentiation of muscle tissue described here and the inhibition of respiration, and if so what is the nature of the connection.

SUMMARY

The injection of cobalt metal powder into the thigh muscle of rats regularly produces a high incidence of characteristic malignant tumours, many of which are derived from the muscle tissue itself. The steps in this process have been followed and found to be very similar in different rats. The carcinogenic process appears to be firstly an extensive and continuing breakdown of the differentiated muscle fibres into free myoblasts, and secondly the transformation of some of these myoblasts into malignant variants. A possible mechanism is suggested.

The author is deeply indebted to his technical assistants, Mrs. Audrey Thompson and Miss Angela Orledge for their skill and patient attention to detail without which this work would not have been possible. He also wishes to thank Dr. Honor B. Fell, F.R.S. and his other colleagues for the many helpful and stimulating discussions which have played their part in the interpretation of these findings.

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COAT COLOUR AND EXPERIMENTAL MELANOTIC TUMOUR PRODUCTION IN THE HAMSTER

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It has been shown that multiple cutaneous melanotic tumours are readily produced in hamsters painted with chemical carcinogens (Della Porta *et al.*, 1956; Shubik, *et al.*, 1956; Horning, 1958; Ghadially, 1959) and that these tumours arise from a network of melanocytes surrounding some of the pilosebaceous follicles (Ghadially and Barker, 1960). Hitherto only the common brown variety (often referred to as the golden) of the Syrian hamster has been employed for cutaneous carcinogenesis. Two other colour varieties, cream and white, are also now commercially available. It was decided to search for melanocytic networks in their skin and attempt to produce melanotic tumours by repeated applications of 9 : 10 dimethyl 1 : 2 benzanthracene (DMBA).

MATERIALS AND METHODS

Normal hamster skin.—The skin of six brown, six cream and six white hamsters aged 6–12 months was examined. In each colour group there were equal numbers of males and females.

Preparation of skin for examination.—After clipping and close shaving, the skin on the flanks and dorsum was removed and pinned out on a piece of cork. The subcutaneous tissues were dissected off and the skin floated on 4 per cent formaldehyde for 24–48 hours. The skin was then removed from its cork mount, dehydrated in alcohol, cleared in xylol and mounted in balsam. In some cases the skin was returned to water stained with haematoxylin and eosin, dehydrated, cleared and remounted in balsam. In such whole mounts of skin melanocytic networks can be easily identified under a low power binocular microscope. This technique is preferable to routine sectioning since it allows the inspection of a large area of skin. Some whole skin preparations were subjected to the dopa reaction, gold chloride and Fontana's silver method for melanin. In each instance results were unsatisfactory as the specimens became too opaque for microscopy.

Histological sections.—Pieces of skin and tumour were fixed in 4 per cent formaldehyde and sectioned and stained with haematoxylin and eosin in the usual manner. Some of these sections were also stained with Fontana's silver method for melanin.

Production of tumours.—Twenty-four brown, 24 cream and 24 white hamsters aged 4–6 months and weighing about 90 g. were used in this experiment. In each colour group there were equal numbers of males and females. The hair on the flanks of all these animals was removed with electric clippers. A 2 per cent solution of DMBA in acetone was applied with a camel hair brush to the skin immediately surrounding the left costo-vertebral spot, since previous experiments

(Ghadially and Barker, 1960) have shown that a majority of melanotic tumours occur in this situation. A constant painting technique was established and adhered to so as to ensure that each animal received approximately the same amount of carcinogen. Animals were painted once a week for a period of 22 weeks and observed for a further period of 9 weeks. The number of melanotic tumours seen in each animal was recorded at weekly intervals during the period of painting and at 2 or 3 weeks interval in the later stages of the experiment.

RESULTS

Normal hamster skin

(a) *Costo-vertebral spot.*—The costo-vertebral spot in the brown and cream variety is well pigmented and easily recognized. In the white variety it is pink and difficult to distinguish from surrounding skin. In whole mounts of skin the C.V. spot of the brown and cream hamster shows large heavily melanized melanocytic networks. In the white variety the melanocytic networks are difficult to distinguish from the surrounding tissues. Their presence is however confirmed by the occurrence of a few melanin granules in the expected site, around the pilosebaceous apparatus in some of the specimens. Thus it would appear that in the white hamster the melanocytic networks are present but are difficult to detect because they are hypomelanotic or at times virtually amelanotic.

(b) *Small pigmented spots.*—In addition to the two large pigmented C.V. spots numerous small pigmented spots have been observed by Ghadially and Barker (1960) in the skin of the brown variety of the Syrian hamster, and it was shown that melanocytic tumours arise from these structures. These small black spots are produced by a collection of melanocytes around some of the pilosebaceous follicles (see Fig. 2 and 3 of Ghadially and Barker, 1960). We were able to confirm the presence of a large number of these structures in all the six brown hamsters we examined, but were able to find only a few small black spots in the six cream hamsters. In the six white hamsters no such spots were detected.

Melanotic tumours

Many melanotic tumours developed in the brown and white varieties but none were seen in the cream variety (Fig. 1). Table I shows the rate at which the tumours appeared in the brown and white varieties. It can be seen that melanotic tumours developed earlier and in greater numbers in the brown than in the white variety. Further it was observed that tumours in the white variety increased in size more rapidly ; thus ultimately the white variety showed fewer but much larger tumours (Fig. 1). It was also noted that both in the brown and white hamster some of the small melanotic tumours regressed after painting had ceased, while others were destroyed by expanding squamous cell carcinomas which developed in some animals. An analysis of our results (Table II) shows that many more melanotic tumours developed in females than in males.

Microscopic examination

Almost all the tumours in the brown variety were abundantly melanized and appeared jet black in colour (Fig. 2) ; only an occasional grey hypomelanotic tumour was found.

TABLE I.—*Rate of Melanotic Tumour Production in Brown and White Hamsters Painted with* 9 : 10 *Dimethyl* 1 : 2 *Benzanthracene*

Time in weeks	Number of surviving animals		Number of animals showing tumours		Total number of tumours		Percentage of survivors showing tumours		Average number of tumours per animal	
	Brown	White	Brown	White	Brown	White	Brown	White	Brown	White
6	24	24	0	0	0	0	0	0	0	0
7	24	24	8	0	8	0	33·3	0	1·0	0
8	24	24	8	0	10	0	33·3	0	1·3	0
9	23	24	8	3	16	4	34·8	12·5	2·0	1·3
10	23	24	16	5	24	7	69·6	20·8	1·5	1·4
11	23	24	16	7	34	15	69·6	29·2	2·1	2·1
12	23	24	17	10	38	21	73·9	41·7	2·2	2·1
13	23	24	17	17	41	47	73·9	70·8	2·4	2·8
14	19	24	18	19	42	60	94·7	79·1	2·3	3·2
15	19	24	18	19	60	59	94·7	79·1	3·3	3·1
16	19	24	18	19	76	58	94·7	79·1	4·2	3·1
17	19	23	19	18	86	55	100	78·3	4·5	3·1
18	19	23	19	18	68	58	100	78·3	3·6	3·2
19	19	23	19	18	87	51	100	78·3	4·6	2·8
20	19	23	19	18	103	55	100	78·3	5·3	3·1
21	19	23	19	18	97	56	100	78·3	5·1	3·1
22	19	23	19	18	75	41	100	78·3	3·9	2·3
24	12	17	12	15	75	41	100	88·2	6·3	2·7
26	12	17	12	15	70	42	100	88·2	5·8	2·8
29	12	17	12	15	93	53	100	88·2	7·8	3·5
31	8	17	8	16	62	53	100	94·1	7·8	3·3

TABLE II.—*Rate of Melanotic Tumour Production in Male and Female Hamsters Painted with* 9 : 10 *Dimethyl* 1 : 2 *Benzanthracene*

Time in weeks	Number of surviving animals		Number of animals showing tumours		Total number of tumours		Percentage of survivors showing tumours		Average number of tumours per animal	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
6	24	24	0	0	0	0	0	0	0	0
7	24	24	3	5	3	5	12·5	20·8	1·0	1·0
8	24	24	3	5	5	5	12·5	20·8	1·7	1·0
9	23	24	5	6	11	9	21·7	25·0	2·2	1·5
10	23	24	12	9	16	15	52·2	36·0	1·3	1·7
11	23	24	13	10	20	29	56·5	41·7	1·5	2·9
12	23	24	15	12	27	32	65·2	50·0	1·8	2·7
13	23	24	17	17	44	44	73·9	70·8	2·6	2·6
14	20	23	18	19	45	57	90·0	82·6	2·5	3·0
15	20	23	18	19	51	68	90·0	82·6	2·8	3·8
16	20	23	18	19	57	77	90·0	82·6	3·2	4·1
17	19	23	17	20	62	79	89·5	87·0	3·6	4·0
18	19	23	17	20	53	73	89·5	87·0	3·1	3·7
19	19	23	17	20	54	84	89·5	87·0	3·2	4·2
20	19	23	17	20	58	100	89·5	87·0	3·4	5·0
21	19	23	17	20	57	96	89·5	87·0	3·4	4·8
22	19	23	17	20	31	85	89·5	87·0	1·8	4·3
24	14	15	12	15	31	85	85·7	100	2·6	5·7
26	14	15	12	15	31	81	85·7	100	2·6	5·4
29	14	15	12	15	41	105	85·7	100	3·4	7·0
31	13	12	12	12	43	72	92·3	100	3·6	6·0

A comparison of the tumours in the brown and white hamsters showed that as a rule the tumours in the white variety contain much less pigment. Very often the tumours in the brown variety were so heavily melanized that no cytological details could be discerned (Fig. 2) unless the section was bleached. No such intensely melanized tumour was produced in any of the white hamsters. Here most of the tumours were distinctly hypomelanotic and a few virtually amelanotic (Fig. 3, 4, 5). However, the architecture and cytology of these tumours in the brown and white hamsters is essentially similar. These tumours are composed of spindle cells and large clear polyhedral cells with an ill-defined cell boundary. A study of the early lesions shows quite clearly that these tumours arise from the melanocytic networks of the small spots in hamster skin. Ghadially and Barker (1960) have already illustrated an early melanotic tumour arising from such a melanocytic network in the brown hamster. Fig. 6 and 7 show a similar early lesion in a white hamster. It can be seen that a hypomelanotic tumour has formed around a group of hair follicles. The tumour is deeply placed in the dermis and is separated by a layer of connective tissue from the epidermis above the tumour.

Epithelial tumours

Besides the melanotic tumours described above, many kerato-acanthomas and some squamous cell carcinomas developed in almost all the brown and white hamsters. In the cream varieties less than half the animals developed a few kerato-acanthomas, and only one carcinoma emerged. These tumours in the cream hamsters started to appear approximately six weeks later than similar tumours in the brown and white varieties. Kerato-acanthomas and carcinomas often started adjacent to the C.V. spot which was destroyed as the tumours enlarged.

DISCUSSION

Ghadially and Barker (1960) have shown that carcinogen-induced melanotic tumours in the brown variety of the Syrian hamster arise from a network of melanocytes surrounding the pilosebaceous follicles in the small pigmented spots of the skin. Such networks are not found in mouse or rabbit skin nor do melanotic tumours, as a rule, arise during cutaneous carcinogenesis in these species. It can be argued that if such networks are a prerequisite to the production of melanotic tumours by chemical carcinogenesis in the hamster, then a correlation between the number of networks and the number of melanotic tumours produced should be demonstrable. Our results show that in the brown variety of Syrian hamster the observable networks are numerous and so are the melanotic tumours produced during cutaneous carcinogenesis. In the cream variety the networks are seen much less frequently and we have failed to produce any melanotic tumours. In the white variety however no networks were detected yet many melanotic tumours were produced. Thus a positive correlation between the number of *observable* networks and the number of melanotic tumours produced was not demonstrated. It seems to us that in the case of the white hamster many melanocytic networks exist but with the techniques employed they have proved undemonstrable because they contain virtually no melanin. Since melanocytic networks are few and widely distributed in hamster skin, it is necessary to examine

a large whole mount of skin to detect them, for the chance of cutting across one of these structures in a histological section taken at random would be indeed small. On the other hand, as noted earlier, a thick whole mount of skin is unsuitable for the application of special staining methods for demonstrating melanocytes. Thus it is obvious that amelanotic melanocytic networks would escape detection by the techniques employed. The fact that many hypomelanotic or virtually amelanotic melanocytic networks exist in the C.V. spot and that many amelanotic and hypomelanotic tumours arise in the white variety lends support to the idea that many small amelanotic networks of melanocytes also occur in the rest of the skin in this variety of the Syrian hamster.

In the case of the cream variety, it would appear that both the marked paucity of small melanocytic networks in the skin and a genetic or strain resistance to the action of the carcinogen is responsible for the failure to produce melanotic tumours, for not only were no melanotic tumours produced but there was also a considerable delay in the production of epithelial tumours, and a markedly poorer final tumour yield.

Both Horning (1958) and Ghadially and Barker (1960) have noted that when the C.V. spot is painted with a carcinogen many melanotic tumours develop in the skin surrounding the C.V. spot, but virtually no such tumours develop from the large melanocytic networks within the spot itself. Indeed only one such tumour arising from the C.V. spot was observed by Ghadially and Barker (1960). The present series of experiments once more demonstrates that the C.V. spot is very resistant to carcinogenic action, for no melanotic tumours were produced from this structure.

Further, many epithelial tumours originated adjacent to the spot, which was later destroyed as a result of enlargement and ulceration of these tumours. Thus at times a fictitious appearance of an ulcerated carcinoma arising in a C.V. spot was created. However, the possibility that an occasional carcinoma may have originated from the C.V. spot itself cannot be excluded, for the design of the experiment did not permit the removal of early lesions for histology. The possible reasons why the C.V. spot is so resistant to carcinogenic action have already been discussed by Ghadially and Barker (1960). The best explanation that can be given at the moment is that the carcinogen is probably very rapidly flushed out of the hair follicles by the large sebaceous glands in the C.V. spot.

SUMMARY

The skin of the brown, cream and white variety of Syrian hamsters was painted repeatedly with DMBA. Many melanotic tumours were produced in the brown and white hamsters but none in the cream variety. Most of the melanotic tumours in the brown hamster produced abundant melanin, but as a rule those in the white variety were hypomelanotic or almost completely amelanotic. It is believed that these poorly pigmented tumours arise from amelanotic melanocytic networks in the hamster skin. Two factors seem to be responsible for the failure to produce melanotic tumours in the cream variety, namely, a paucity of melanocytic networks and a genetic or strain resistance to the action of the carcinogen.

We are indebted to Professor D. H. Collins for helpful advice and criticism, to Mr. T. L. Platts and Miss S. Wall for photomicrographs and to Mr. J. H. Carver

and Mrs. A. Whitaker for technical assistance. This work was supported by grants from the University of Sheffield Medical Research Fund and the British Empire Cancer Campaign.

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EXPLANATION OF PLATES

FIG. 1.—Brown, cream and white hamsters painted with DMBA. Melanotic tumours have developed in the brown and white variety only. $\times\frac{3}{4}$.

FIG. 2.—Typical abundantly melanized melanotic tumor in a brown hamster. H. and E. $\times 7{\cdot}5$.

FIG. 3.—Hypomelanotic tumour from a white hamster. Bulk of the tumour cells contain little or no melanin. Only a few densely pigmented cells are scattered throughout the tumour. H. and E. $\times 75$.

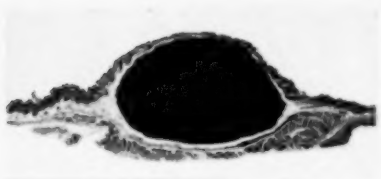
FIG. 4.—High power view from tumour illustrated in Fig. 3, showing a few well-pigmented cells scattered among cells containing little or no melanin. H. and E. $\times 666$.

FIG. 5.—An almost completely amelanotic melanoma from a white hamster. In many serial sections only an occasional granule of melanin could be detected. Silver staining revealed a few more. H. and E. $\times 666$.

FIG. 6.—An early lesion illustrating a hypomelanotic tumour arising from a melanocytic network surrounding the pilosebaceous follicles in a white hamster. Note that the tumour is deeply placed and has no connection with the epidermis. H. & E. $\times 55$.

FIG. 7.—High power view from tumour illustrated in Fig. 6. The cells are in close contact with the hair follicle seen on the left of the photomicrograph. The tumour has grown as a sheath enveloping a group of pilosebaceous follicles. H. & E. $\times 330$.

Fig. 1.



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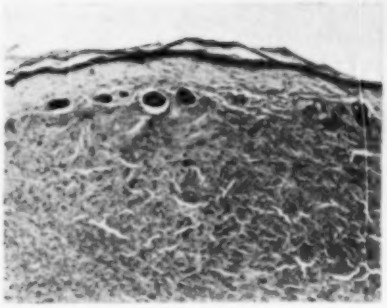

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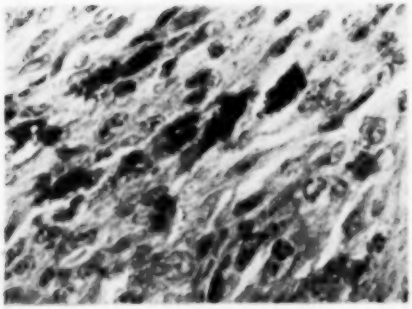

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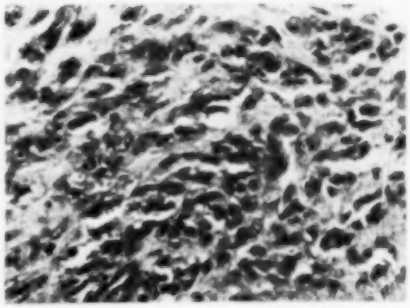

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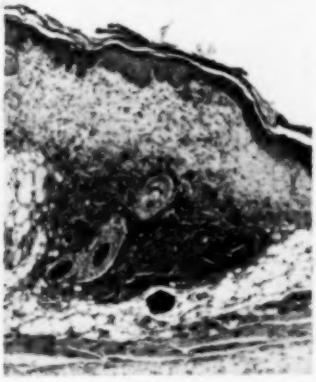

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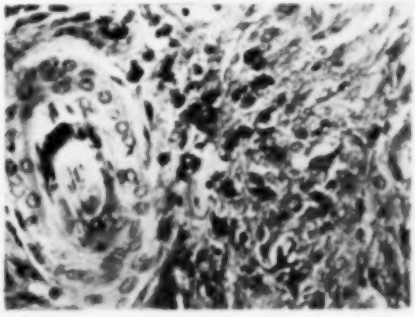

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THE EFFECT OF ENDOCRINE CHANGES, OF IRRADIATION AND OF ADDITIONAL TREATMENT OF THE SKIN ON THE INDUCTION OF TUMOURS IN THE FEMALE GENITAL TRACT OF RATS BY CHEMICAL CARCINOGENS

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CHANGES in the hormonal environment affect the development of tumours of endocrine organs (Gardner, 1953; Kirschbaum, 1957; Bielschowsky and Horning, 1958) and also the induction of tumours by chemical carcinogens in the breast (Mühlbock, 1956; Huggins, Briziarelli and Sutton, 1959), liver (Griffin, Rinfret and Corsigilia, 1953) and skin (Marchant, 1959). Huggins, Torralba and Mainzer (1956) and Huggins *et al.* (1959) have shown that the ovary, the pituitary and the adrenals influence the growth of transplanted tumours and the induction of breast cancers by the ingestion of methylcholanthrene in rats. In a previous report (Glucksmann and Cherry, 1958) we have indicated that castration reduces the incidence of vaginal sarcomas in rats after the intravaginal application of 9, 10-dimethyl-1, 2-benzanthracene (DMBA) but does not affect the production of carcinomas of the vulva. Administration of oestrogen only partially succeeded in increasing the tumour incidence in the vagina.

The report of the present series of experiments pursues the theme further in an attempt to ascertain the influence of hormonal stimulation and of ablation of endocrine organs on the formation of tumours in the female genital tract of rats. Adrenalectomy was added to ovariectomy in the expectation of a further reduction of tumour incidence, progesterone was given to spayed rats and the influence of forced breeding was tested. The effect of repeated pelvic irradiation as a means of castration was investigated and was also studied after surgical castration for its influence on tumour production. Single and repeated whole body exposures to irradiation are known to increase the rate of tumour production in various organs (Glucksmann, Lamerton and Mayneord, 1957), to promote tumour formation in combination with chemical carcinogens and hormones (Kirschbaum, 1957) but also to delay slightly the emergence of tumours at other sites (Lisco, Ducoff and Baserga, 1958). Repeated whole body exposures were given to rats in addition to the intravaginal application of carcinogens to study the effect of X-rays on tumour formation.

Topical application of chemical carcinogens increases the incidence of tumours of the lung (Shimkin, 1955), the ovaries (Howell, Marchant and Orr, 1954) and the breast (Huggins *et al.*, 1959; Geyer, Bryant, Bleisch, Peirce and Stare, 1953). It was thus thought worth while to examine the effect of additional applications of the chemical carcinogen to the dorsal skin in addition to its intravaginal administration. Further experiments on the effect of combined administration of oestrogen and progesterone to spayed rats, of thyroxin to castrated animals and of thiouracil to virgin rats are still in progress.

MATERIAL AND METHODS

Female black-hooded rats inbred in this laboratory were used when 2 to 3 months old. The experimental animals were painted intravaginally with a 1 per cent solution in acetone of 9, 10-dimethyl-1, 2-benzanthracene (DMBA) obtained from Messrs. L. Light & Co., while the controls were painted with acetone. The application was by means of a cotton wool swab on the end of a galvanized wire. The vagina was stretched open by dorsal flexion of the tail, the swab was inserted and the vagina and cervix painted by means of a rotary motion. This form of administration entailed contamination of the vulva which was reduced in some experiments (Table I) by blotting the vulva with filter paper immediately after the painting.

TABLE I.—*Experimental Procedures and Number of Animals*

State of Rat	Additional treatment	Number of weekly paintings		Number of rats	Remarks
Virgin	*Nil*	1·5*	DMBA	28	—
Virgin	,,	1·5*	Acetone	12	—
Castrated surgically	,,	2	DMBA	16	—
idem	,,	2	Acetone	8	—
,,	oestrogen, 2 × weekly	2	DMBA	16	—
,,	progesterone 2 × weekly	1	DMBA	31	Vulva blotted
,,	,,	1	Acetone	12	,,
,,	adrenalectomy	1	DMBA	16	,,
,,	,,	1	Acetone	8	,,
,,	,,	0	none	4	—
,,	6 × 340 r to pelvis in 20 weeks	2	DMBA	16	—
,,	,,	2	Acetone	8	—
Virgin	,,	2	DMBA	16	—
Virgin	,,	2	Acetone	8	—
Pregnant	*Nil*	1	DMBA	24	Vulva blotted
Virgin	4 × 400 r whole body in 30 weeks	1	DMBA	21	—
Virgin	DMBA to dorsal skin 1 × weekly	1	DMBA	21	—

* Once weekly for 28 weeks and then twice weekly for 27 weeks.

Surgical procedures.—Bilateral ovariectomy was performed under ether anaesthesia at the age of 2 to 3 months and the painting was started 1 to 2 months later. Bilateral adrenalectomy was performed under ether anaesthesia 3 weeks after ovariectomy and painting started 1 month later. These rats were kept on saline for the duration of the experiment.

Radiation procedures.—A pelvic field including the vagina, cervix, uterus, ovaries and adrenals was irradiated through a heart-shaped hole of 20 cm.^{2} in a lead shield of 1 cm. thickness. The animals were held in position by means of a plastic cloth clamped to a metal holder. A dose of 340 r. was given in 4 minutes and repeated at intervals of 4 weeks over a period of 20 weeks to a total dose of 2040 r in 6 exposures. Radiation factors were: 200 KV X-rays at 10 mA, focal skin distance 25 cm., filtration 1·0 mm. Al and 1·0 mm. Cu.

For whole body exposure the animals were placed in a plastic box of 20 × 20 cm. and irradiated from below. A dose of 400 r was given in 9 minutes and the exposures repeated at intervals of 10 weeks over a period of 30 weeks to a total dose of 1600 r in 4 exposures. Radiation factors were: 200 KV X-rays at 10 mA, focal skin distance 50 cm., filtration 1·0 mm. Al and 0·5 mm. Cu.

Additional treatments.—To maintain an almost continuous state of pregnancy 4 females were housed in a cage with 1 male and the litters removed from the cage soon after birth. This arrangement gave up to 11 litters per rat in 10 months, though some rats proved to be less fertile (Fig. 3).

A dose of 1 μg. of oestradiol monobenzoate (Organon, Ltd.) in olive oil was injected intramuscularly twice weekly.

Progesterone (Organon, Ltd.) also was given twice weekly intramuscularly in a dose of 1 mg.

The dorsal skin region in one experiment was painted with a cotton wool swab with DMBA in the same concentration as was used for the vagina.

The experiments were performed over a period of 4 years, but there was an overlap of several months between the different groups of experiments and within each group the controls were carried out at the same time.

Histological Methods.—Animals were killed when definite evidence of the presence of a vaginal tumour was available or when extensive vulval tumours or other conditions made it necessary. The rats were inspected at least once weekly and notes made of macroscopic lesions such as warts.

At autopsy the uterus, cervix, vagina, vulval skin and the ovaries (except in surgical castrates) were fixed in Zenker-acetic or in the Susa fixative, dehydrated and embedded in paraffin, sectioned at 8μ. and the sections stained with haematoxylin-eosin, by the periodic acid-Schiff technique with or without previous diastase digestion, by the Feulgen method, with Southgate's mucicarmine stain, with van Gieson's stain or with a modified " Azan " stain.

Where the dorsal skin was painted, this was excised for fixation. Adrenals were fixed routinely and a special search was made for remaining adrenal tissue in rats in which an adrenalectomy had been performed.

The thickness of the uterine and vaginal stroma was measured histologically as the distance between the innermost muscle layer and the basement membrane of the epithelium.

RESULTS

Tumour induction in the vagina

The majority of vaginal tumours were sarcomas arising in the subepithelial stroma. Presarcomatous lesions and fibromas developed less frequently in the same localization while epithelial tumours such as papillomas and carcinomas occurred only rarely.

The sarcomas were cellular with varying amounts of fibre formation, often with multi- or mononucleate giant cells and sometimes with a leiomyomatous component. They spread into the cervix, uterus and vulva and involved the neighbouring pelvic structures growing along the perineural lymphatics. There was no evidence of metastatic spread but this is not surprising since the animals were killed at the first sign of local malignancy.

The presarcomatous lesions were characterized by the appearance of very large, abnormal cells in perivascular infiltrates surrounded by fibre forming fibroblasts (Glucksmann and Cherry, 1958). Fibromatous thickening of the subepithelial stroma of the vagina was found in 3 animals.

The vaginal epithelium reacted to the application of DMBA with only a mild hyperplasia, though the degree varied in different experiments, leading in some

to the appearance of warts or of carcinomas in the middle or posterior half of the vagina.

TABLE II.—*Vaginal Tumours Induced by DMBA-painting*

State of rat	Additional treatment	Number at risk	Sarcomas		Presarcomas		Fibromas		Carcinomas		Warts		All tumours		Number of rats with vaginal tumours	
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Castrate	*Nil*	15	3	20	0	0	0	0	0	0	0	0	3	20	3	20
Virgin	Whole body X-rays	20	6	30	0	0	0	0	0	0	0	0	6	30	6	30
Virgin	Pelvic X-rays	16	5	31	3	19	0	0	0	0	0	0	8	50	8	50
Castrate	Oestrogen	16	5	31	3	19	0	0	0	0	0	0	8	50	8	50
Castrate	Progesterone	28	12	43	1	4	0	0	0	0	0	0	13	47	13	47
Virgin	DMBA to skin	21	7	33	0	0	2	10	0	0	0	0	9	43	9	43
Virgin	*Nil*	23	16	70	0	0	0	0	1	4	1	4	18	78	16	70
Pregnant	*Nil*	23	18	78	2	9	0	0	0	0	3	13	23	100	20	87
Castrate	Pelvic X-rays	15	11	73	0	0	0	0	2	13	1	7	14	93	14	94
Castrate	Adrenalectomy	8	6	75	0	0	0	1	12	0	1	12	8	100	8	100

Table II gives the incidence of tumours after the different treatments. None of the acetone-painted controls (Table I) developed any tumours in the vagina or the vulva. Animals "at risk" survived the first painting for at least 150 days. The incidence of tumours ranged from 20 to 100 per cent and three levels can be distinguished. At the lowest (20–30 per cent) were the rats painted after surgical castration or castration by repeated whole body irradiation. At an intermediate level of tumour incidence (43–50 per cent) were (a) virgin rats subjected to pelvic irradiation, (b) surgical castrates given oestrogen, (c) surgical castrates given progesterone and (d) virgin rats whose dorsal skin was painted with DMBA. The incidence of sarcomas of the vagina is the same in the low and intermediate group, but in the latter fibromas and presarcomatous lesions are found in addition to sarcomas and raised the total tumour incidence. The highest level of tumour incidence was achieved by (a) virgin rats, (b) pregnant rats, (c) surgical castrates subjected to repeated pelvic irradiation and (d) surgical castrates with bilateral adrenalectomy. In this group only were found papillomas and carcinomas of the vagina (Table III), giving rise to multiple tumours (carcinoma or wart plus sarcoma) in the same rat. In this group the incidence of tumours exceeded that of tumour bearing rats (Table II) while the two figures are identical at the lower levels of tumour incidence.

The difference in total tumour induction between the three levels is significant at the 95 per cent confidence level. The high and intermediate tumour groups

TABLE III.—*Type of Vaginal Tumours at Three Different Incidence Levels*

Incidence level	Sarcomas %	Presarcomas + fibromas %	Carcinomas + papillomas %	All tumours %
Low	26 ± 7·4	0	0	26 ± 7·4
Intermediate	37 ± 5·4	10 ± 3·5	0	47 ± 5·5
High	74 ± 5·3	4 ± 2·4	13 ± 4·0	91 ± 3·4

also differ significantly in the incidence of sarcomas while there is no significant difference in this respect between the low and the intermediate level. There is also a significant fall in the proportion of presarcomatous and fibromatous lesions to sarcomas in the progression from the intermediate to the high level : i.e. there are 9 non-malignant lesions and 29 sarcomas in the intermediate group, but only 3 non-malignant lesions and 58 sarcomas in the high incidence group (Table II).

Time ranges in tumour development

Since animals were killed at the first definite indication of vaginal tumours or when large vulval growths or other conditions made it necessary, the true induction time could not be determined. As the tumours varied in size when first discovered, no reliable estimate of the period of growth can be given. For the same experiment the average " survival " time of rats with sarcomas and those without was not statistically different except for surgical castrates given pelvic irradiation (Table IV). There are marked differences, however, between the

TABLE IV.—*Average Survival Period for Rats with and Without Vaginal Sarcomas After DMBA Painting*

State of rat	Additional treatment	Survival in days of rats: With sarcoma	Without sarcoma	Difference
Castrate	*Nil*	328 ± 46·1	373 ± 17·0	45 ± 49·1
Virgin	Whole body X-rays	296 ± 19·7	310 ± 15·5	14 ± 25·0
Virgin	Pelvic X-rays	248 ± 9·4	239 ± 9·9	9 ± 13·6
Castrate	Oestrogen	341 ± 33·3	324 ± 14·8	17 ± 36·5
Castrate	Progesterone	260 ± 25·1	298 ± 38·1	38 ± 45·6
Virgin	DMBA to skin	270 ± 3·9	257 ± 11·2	13 ± 11·8
Virgin	*Nil*	337 ± 8·0	311 ± 16·4	26 ± 18·2
Pregnant	*Nil*	246 ± 9·9	250 ± 21·1	4 ± 23·4
Castrate	Pelvic X-rays	217 ± 7·2	184 ± 2·0	33 ± 7·5
Castrate	Adrenalectomy	295 ± 38·8	227 ± 42·5	68 ± 57·5

various experimental groups (Fig. 1). In the high tumour incidence group the speed of tumour development was the fastest in 3 of its 4 members, while the fourth (virgins painted with DMBA) lagged behind the 3 fastest members of the intermediate group. The speed of tumour development was slowest in the low incidence group. In this as well as in the intermediate group even some of the oldest animals failed to form tumours.

If the average speed of tumour development at the three levels is considered (Fig. 2), there is a clear tendency for a shift to the right suggesting a correlation between speed and incidence of tumour formation. It remains obscure, however, why at the same level of tumour incidence the speed differed between the various experimental series. Seasonal variations are not likely to account for the difference, since the experiments overlapped in time.

Sarcomas and presarcomatous lesions appeared at about the same time. In the experiment with forced breeding (Fig. 3) the two presarcomatous lesions appeared at fairly late intervals. This figure illustrates also the relation between the number of litters and the appearance and type of tumours in the pregnant rats. Of the three animals that failed to produce tumours, one had the lowest

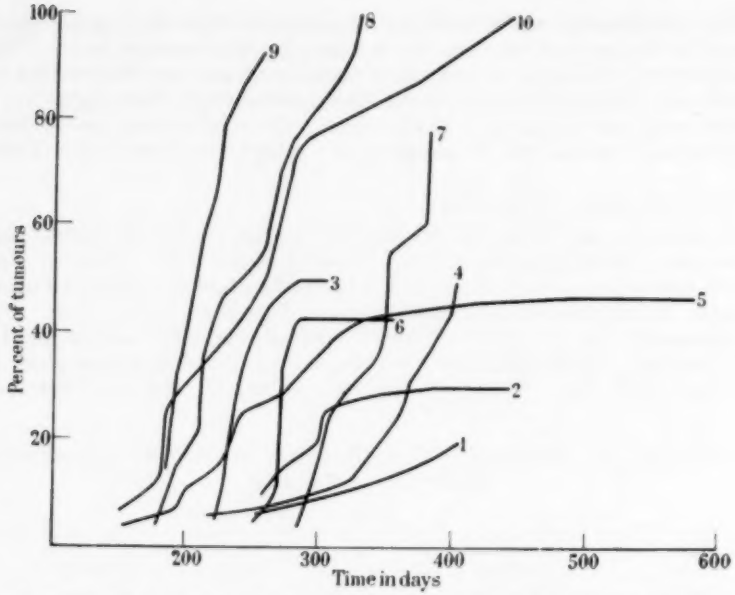

FIG. 1.—Cumulative percentage of all vaginal tumours for different treatments additional to painting with DMBA : (1) castration, (2) whole body X-rays, (3) pelvic X-rays, (4) castration plus oestrogen administration, (5) castration plus progesterone treatment, (6) painting of dorsal skin with DMBA, (7) virgin rats, (8) forced breeding, (9) castration plus repeated pelvic X radiation, (10) castration plus adrenalectomy.

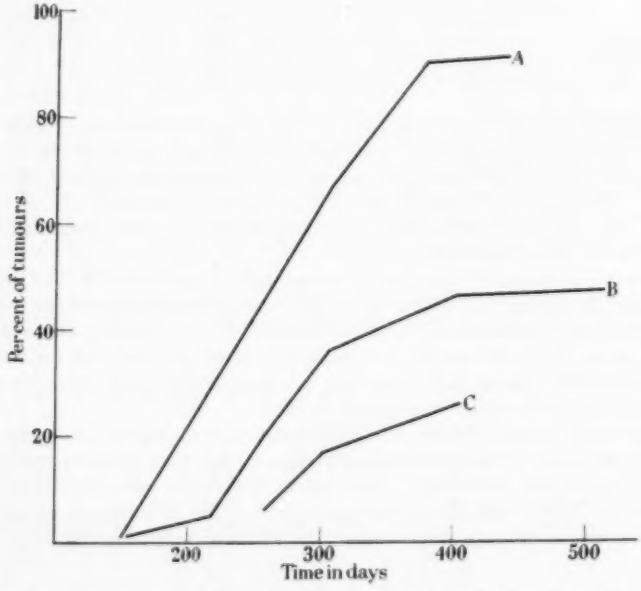

FIG. 2.—Cumulative percentage of all vaginal tumours in high (A), intermediate (B) and low (C) tumour incidence group.

number of litters, while the other two had 7 or 8 litters like the majority of tumour bearing rats. Of the presarcomatous lesions one occurred in a highly fertile rat.

The relation of uterine and vaginal activity to carcinogenesis

Castration by surgery, by whole body and to a lesser extent by pelvic irradiation reduced the incidence of vaginal tumours and caused atrophy of the uterus. Histological measurements of the thickness of the uterine and vaginal stroma in Table V show that the hormonal influence on tumour formation was not closely correlated with that on the growth of the normal tissue. Thus the uterine stroma remained atrophic in surgical castrates subjected to pelvic irradiation or to adrenalectomy while the tumour production was greatly increased. Conversely the

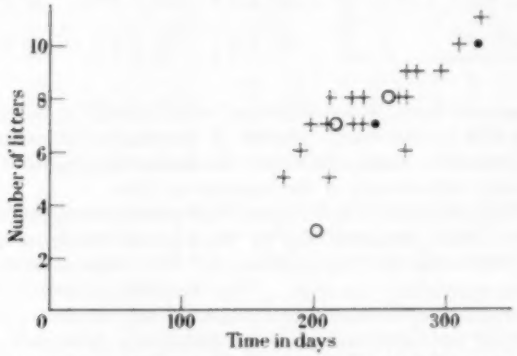

FIG. 3.—Number of litters and tumour incidence in individual rats painted with DMBA and subjected to forced breeding.

○ = no tumour, + = sarcoma of the vagina, ● = presarcomatous lesion.

painting of the dorsal skin did not reduce the thickness of the uterine and vaginal stroma, but decreased tumorigenesis in the vagina.

The secretory activity of the endometrial epithelium and its glands was reduced by castration, restored by oestrogen or progesterone treatment and less regularly by adrenalectomy. The last procedure failed to affect the atrophy of the uterine stroma and progesterone had only a slight effect on it. Thus even normal tissues, i.e. stroma and epithelial components of the uterus, react in a different manner to hormonal stimulation and neither shows a consistent correlation with the liability to tumorigenesis.

The same applies to the vaginal stroma which though reduced by castration did not show a correlation of its width with tumour formation (Table V). This finding confirms previous observations (Glucksmann and Cherry, 1958) and the conclusion that the vaginal stroma responds to oestrogenic stimulation but to DMBA only locally and not generally.

The squamous hyperplasia of the vaginal epithelium induced by DMBA painting was diminished by surgical spaying and by castration with whole body irradiation. Progesterone produced a high cornifying epithelium while oestrogen restored only partially the hyperplasia lost on castration. In the pregnant rat the state of hyperplasia and the type of epithelium varied with the stage of preg-

TABLE V.—*Incidence of Vaginal Tumours and Width of Uterine and Vaginal Stroma in DMBA-treated and Control Rats*

State of rat	Additional treatment	Tumours %	Width of uterine stroma: DMBA painted	Width of uterine stroma: Control	Width of vaginal stroma: DMBA painted	Width of vaginal stroma: Control
Castrate	*Nil*	20	32 ± 4·4	32 ± 3·7	15 ± 2·6	14 ± 2·5
Virgin	Whole body X-rays	30	43 ± 8·3	—	17 ± 1·9	—
Virgin	Pelvic X-rays	50	46 ± 15·7	32 ± 5·2	21 ± 2·6	19 ± 3·3
Castrate	Oestrogen	50	83 ± 7·2	—	23 ± 2·5	—
Castrate	Progesterone	47	39 ± 4·04	39 ± 4·1	19 ± 2·1	20 ± 2·2
Virgin	DMBA to skin	43	70 ± 6·7	—	25 ± 2·3	—
Virgin	*Nil*	78	81 ± 8·8	80 ± 10·0	28 ± 3·9	29 ± 1·1
Pregnant	*Nil*	100	—	—	—	—
Castrate	Pelvic X-rays	93	35 ± 6·7	30 ± 3·2	21 ± 3·1	17 ± 1·8
Castrate	Adrenalectomy	100	27 ± 3·1	28 ± 6·3	16 ± 0·5	18 ± 1·4

nancy : at and around term, the epithelium was formed by mucin secreting, high columnar cells while in the early phases of pregnancy hyperplastic squamous epithelium was present. Irrespective of the stage of pregnancy the epithelium overlying a sarcoma was always of the squamous type.

The degree of hyperplasia of the vaginal epithelium was greatest near epithelial tumours and since these occurred only in the highest incidence group, there was some correlation between the hyperplasia of the vaginal epithelium and the tendency to form epithelial tumours. This applied, however, to the localized rather than generalized epithelial hyperplasia of the vagina. A relatively high epithelium occurred in castrates after progesterone treatment and in virgins treated with painting of the dorsal skin, but in neither of these groups were epithelial tumours found. Progesterone also stimulated the cervical epithelium, and the high columnar cells at this site contrasted strongly with the squamous epithelium of the vagina.

Adrenal remnants were found in 7 of the 8 tumour-bearing adrenalectomized rats. They consisted of abnormal looking cortical tissue lying in the fat close to the capsule of the kidney. The functional activity of these structures could not be assessed, but the rats had to be maintained on saline solution and lost weight immediately on being put on tap water. It cannot be decided whether these remnants are due to the regeneration of cortical tissue left behind at operation or to the hypertrophy of small foci of accessory cortical tissue in the rats. These remnants were also found in the controls treated with acetone and tended to increase in size with time after adrenalectomy.

The ovaries of rats in which the dorsal skin as well as the genital tract were painted with DMBA showed no abnormalities except for one animal in which the ovaries were largely replaced by cysts. This rat had no vaginal tumours, but a carcinoma and a sarcoma in the dorsal skin and a papilloma on the vulva.

Tumour induction in the vulva

All but one of the 145 vulval tumours were derived from the epithelium and 71 per cent were squamous cell carcinomas while 28 per cent were squamous or basal-celled papillomas. Only one sarcoma was found at this site. The vulval skin showed very marked hyperplastic changes after DMBA painting (Glucksmann

and Cherry, 1958) and the degree of hyperplasia as well as the incidence of tumours was independent of castration, hormonal treatments and irradiation, but was considerably reduced though not entirely suppressed by the blotting of the vulva immediately after painting (Table VI). Tumour induction in the vulva thus

TABLE VI.—*Tumours of the Vulva Induced by DMBA-painting*

State of rat	Additional treatment	Number at risk	Carcinomas No.	Carcinomas %	Papillomas No.	Papillomas %	Sarcomas No.	Sarcomas %	All tumours No.	All tumours %	Number of weekly paintings
Castrate	*Nil*	14	11	78	1	8	0	0	12	86	2
Virgin	Whole body X-rays	20	17	85	3	15	0	0	20	100	1
Virgin	Pelvic X-rays	16	14	88	2	12	0	0	16	100	2
Castrate	Oestrogen	16	9	56	4	25	0	0	13	81	2
Castrate	Progesterone	28	2	7	5	18	0	0	7	25	1*
Virgin	DMBA to skin	21	16	76	4	19	1	5	21	100	1
Virgin	*Nil*	23	15	65	6	26	0	0	21	92	1·5
Pregnant	*Nil*	23	5	22	10	43	0	0	15	65	1*
Castrate	Pelvic X-rays	15	11	73	4	27	0	0	15	100	2
Castrate	Adrenalectomy	8	3	37	2	25	0	0	5	62	1*

* = Vulva blotted with filter paper after painting of vagina.

appears to be independent of hormonal state but to vary with dosage of the carcinogen at certain dosage levels. Single applications per week induced as many tumours as two applications per week. Only blotting after single weekly applications reduced the tumour incidence.

The speed of tumour formation varied markedly between the different experiments as seen in Fig. 4, in which the cumulative percentage of tumours is plotted against the time of histological confirmation. Table VII gives the interval be-

TABLE VII.—*Incidence of Tumours at the Vulva and Time to Appearance of First Wart After DMBA-painting of the Vagina*

State of rat	Additional treatment	Tumours %	First wart days
Castrate	*Nil*	86	265 (248)
Virgin	Whole body X-rays	100	154
Virgin	Pelvic X-rays	100	135
Castrate	Oestrogen	81	136
Castrate	Progesterone	25*	193
Virgin	DMBA to skin	100	112
Virgin	*Nil*	92	203
Pregnant	*Nil*	65*	126
Castrate	Pelvic X-rays	100	135
Castrate	Adrenalectomy	62*	269

* = Vulva blotted after vaginal painting.

tween the beginning of painting and the appearance of the first warts on the vulva. In general warts were noticed 40 to 60 days before the histological confirmation, but in one experiment on surgically castrated rats a basal cell tumour was found on histological examination 20 days before the first macroscopic warts appeared. The basal cell tumours could not be spotted macroscopically as they grew inwards rather than outwards.

The shortest induction period for warts occurred in rats whose skin also was painted with DMBA, though even here papillomas in the dorsal skin preceded the vulval warts by 42 days. This difference may have been due to dosage of the carcinogen or to the size of the painted field which is of necessity small for the

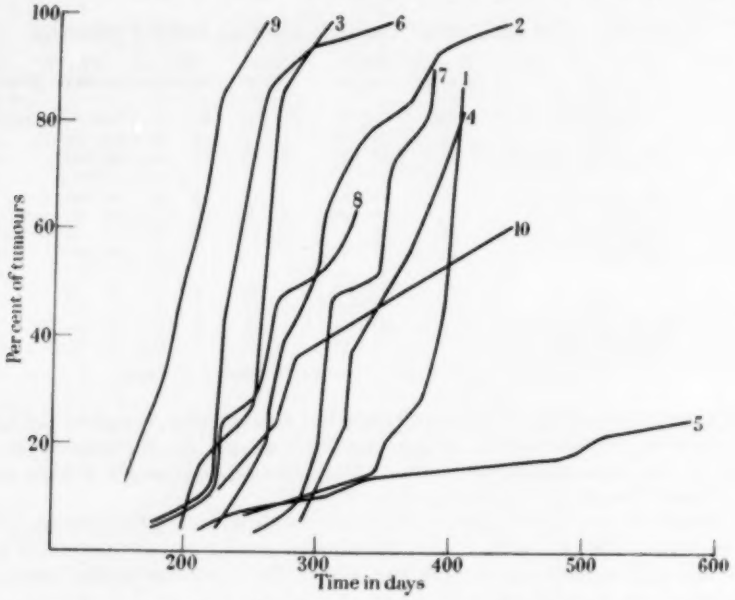

FIG. 4.—Cumulative percentage of all tumours at the vulva after different treatments: (1) castration, (2) whole body X-rays, (3) pelvic X-rays, (4) castration plus oestrogen, (5) castration plus progesterone treatment and blotting of vulva, (6) painting of dorsal skin with DMBA, (7) virgin rats, (8) forced breeding plus blotting of vulva, (9) castration plus pelvic radiation, (10) castration, adrenalectomy and blotting of the vulva.

vulva. Since in pregnant rats the first warts appeared early in spite of the blotting after the painting, it is doubtful whether dosage of the carcinogen hastened the formation of tumours. On the other hand the proportion of papillomas to carcinomas was significantly greater when the vulva was blotted (17 warts of 27 tumours or 63 per cent) than when this procedure was omitted (24 warts of 117 tumours or 21 per cent). Thus the degree of malignancy as well as the total incidence of tumours decreases with the reduction of contact with the carcinogen, but the induction time for the first warts is not lengthened.

DISCUSSION

The tumour incidence in the vulva and vagina is the same whether the DMBA is applied once or twice a week and thus is independent of dose at this level of carcinogenic stimulation. The incidence particularly of malignant vulval tumours is decreased by blotting after single weekly applications of DMBA and at this dose level tumour development of the vulva is obviously dependent on dose. It is noteworthy that in the vulva as in the vagina the increase in total tumour inci-

dence is accompanied by an increase in the proportion of malignant lesions (Table III).

The systemic factors tested by the additional treatments affect the incidence of vaginal but not of vulval tumours and thus we have to consider the incipient neoplastic tissue of the vagina as susceptible to systemic and in particular to hormonal action while the vulval skin is not. The response of the tumour-forming tissue of the vagina to hormones differs from that of the normal stroma of the vagina from which it is derived and also from that of the uterus of the same animal. Thus the tumour incidence does not vary with the width of the vaginal or uterine stroma (Table V) which responds to oestrogenic treatment. Similarly Huggins *et al.* (1956) found a difference in the response of a transplanted fibroadenoma and that of the normal breast to hormonal stimulation in ovariectomised rats. Progesterone accelerated the growth of the tumour but not that of the breast. Oestrogen stimulated the growth of the breast irrespective of dose and the growth of the tumour in small doses, but retarded it in large doses. In our experiments a dose of oestrogen sufficient to restore fully the width of the uterine and vaginal stroma of castrates failed to restore the tumour incidence though it increased it. The dose of oestrogen used was in the lower dose range which in Huggins's experiments still had a stimulating effect on the fibroadenoma and promoted tumour induction in the breast (Huggins *et al.*, 1959). The effect of oestrogen on tumour production in our experiments was of the same order as that of progesterone but in Huggins *et al.*'s experiments much larger doses of progesterone promoted the induction of breast tumours very much. The difference in the results may thus be attributable to dosage, rather than to a difference in reactivity of the breast and the vagina.

Surgical castration and castration by repeated whole body irradiation greatly reduced the tumour incidence in the vagina but did not completely abolish it (Table III, low level). A similar effect of surgical castration on the induction of breast tumours has been reported by Huggins *et al.* (1959). The administration to ovariectomised rats of progesterone or oestrogen induced presarcomatous and fibromatous lesions and did not greatly increase the incidence of vaginal sarcomas (Table III, intermediate level). Tumour incidence was also reduced by the slow castration of rats through repeated pelvic irradiation and by the painting of the dorsal skin with DMBA. The latter procedure produced no ovarian tumours and ovarian cysts occurred in only one rat which failed to develop a vaginal tumour. The measurements on the vaginal and uterine stroma do not suggest a deficiency in the oestrogenic stimulation in these rats. In this respect the experimental results resemble closely those obtained with oestrogen administration to ovariectomised rats. It is significant that in this intermediate group of tumour incidence the increase is due mainly to the appearance of presarcomatous and fibromatous lesions while in the high incidence group the increase is due to sarcomas and the non-malignant lesions become rare (Table III, high level).

In this high incidence group belong the virgin and the pregnant rats and also the surgical castrates with adrenalectomy and with repeated pelvic irradiation. The high tumour incidence in the virgin and pregnant rats is not unexpected but that in the other two groups is surprising. Adrenalectomy of ovariectomized rats increases the retardation in the growth of transplanted fibroadenomas (Huggins *et al.*, 1956), and slows down the growth of Walker tumours (Slawikowski, 1960) and was performed by us with the expectation of totally suppressing the

induction of vaginal sarcomas. The remarkable promotion of tumorigenesis may be due to the persistence of possibly active adreno-cortical remains. Even then some abnormality in the function of the adrenal must be assumed. It is feasible that an abnormal function of the adrenals is also responsible for the high tumour incidence in spayed rats receiving X-rays over a pelvic field including the adrenals. Ovariectomy may be followed by the hyperplasia of the adrenal cortex and even tumour formation (Bielschowsky and Horning, 1958) in response to high levels of pituitary gonadotrophin after castration. Irradiation of the adrenals in such a state but not in the intact animal may produce an abnormal secretion able to promote tumour formation in the vagina but without effect on the width of the uterine and vaginal stroma.

The complexity of the hormonal interactions is also illustrated by the experiments of Shay, Harris and Gruenstein (1952) in which breast tumours were induced in male and female rats by the administration of methylcholanthrene by stomach tube. Castration of females greatly reduced the tumour incidence but testosterone and progesterone only slightly lowered the tumour incidence in intact females. On the other hand oestrogen treatment much increased the tumour incidence in intact and castrated males. The interplay of various endocrines is also brought out by Huggins *et al.* (1956) who also showed that hypophysectomy inhibits the growth of the transplantable fibroadenoma, that oestrogen alone had hardly any effect on the tumour growth in such animals, that the combination of oestrogen and progesterone was more effective and that this effect was still further increased by the addition of lactogenic hormone or growth hormone. In our experiments castration decreases the tumour incidence and width of the vaginal and uterine stroma ; the administration of oestrogen increases the width of the uterine and vaginal stroma, but only slightly enhances the tumour formation in the vagina ; adrenalectomy and pelvic irradiation significantly increase the incidence of tumours without stimulating the growth of the uterine and vaginal stroma.

It is quite obvious that the endocrine and systemic influences discussed here are not of generalized nature, i.e. they do not affect all tissues alike but exert their action only on specific target tissues. Whether they act as sensitizers to the initiating action of DMBA or promote the growth of changed cells, cannot be decided. It is noteworthy, however, that the non-malignant forms appear first at the intermediate level of tumour induction and that only at the highest level of induction do the epithelial tumours appear in addition to the increased number and proportion of sarcomas. Though on the average the speed of tumour formation is also increased in the same direction (Fig. 2), the differences in individual experiments (Fig. 1) show that the rate and speed of tumour induction are not necessarily closely linked with one another.

The absence of a correlation between the width of the uterine and vaginal stroma and the tumorigenesis shows that the systemic influences cannot be considered as general promoters of growth in the form of mitotic stimuli. The fact that castration decreases the tumour incidence and oestrogen slightly increases it is evidence against the hypothesis of Jackson and Robson (1957) that oestrogenic hormones compete with carcinogens for the specific growth receptors. It seems rather that specific levels of reactivity to DMBA in the tissues of the target organs and of their stimulation by as yet undefined endocrine agents have to be postulated to explain the differential behaviour of vaginal stroma and vaginal epithelium

in tumour formation. The vulva shows such a high responsiveness to DMBA that an influence of systemic factors has been obscured if it exists at all.

SUMMARY

Ovariectomy reduced the incidence of vaginal tumours after intravaginal application of DMBA, and administration of oestrogen or of progesterone raised the incidence of tumours only slightly.

Repeated whole body exposures to X-rays also lowered the rate of tumour incidence after painting and so to a lesser extent did repeated pelvic irradiation of virgin rats and the application of DMBA to an additional dorsal skin region.

In surgical castrates adrenalectomy or repeated pelvic irradiation restored the level of tumour incidence to that of intact and pregnant rats.

Three levels of vaginal tumour incidence are found and the distribution of tumour types and the length of the average induction time varied with the level: at the lowest level there are only sarcomas, at the intermediate level fibromas and presarcomatous lesions are found in addition to the sarcomas while at the highest level the incidence of sarcomas is increased and epithelial tumours appear.

Tumour induction in the vulva is not affected by castration, radiation or hormone treatment but varies at certain dose levels with the dose of the carcinogen.

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REDUCTION OF MAMMARY CARCINOMA AND ADENOMA IN C_3Hf BREEDERS AFTER LATE OVARIECTOMY

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THE earliest reports of reduction in mammary carcinoma after ovariectomy were made in inbred virgin female mice at various ages (Lathrop and Loeb, 1916; Loeb, 1919; Cori, 1926, 1927) and in former DBA breeders (Murray, 1928, 1932, 1936). These experiments preceded the discovery of the mammary tumour agent by Bittner in 1936. On account of the high spontaneous incidence of this tumour in the mouse strains used at that time and the evidence obtained since then of infection of DBA mice with the agent, it is probable that all reported reductions occurrred in infected stocks. Later observations, which have confirmed earlier results, have been made with C_3H mice known to carry the agent (Shimkin and Wyman, 1945; Pilgrim, 1957). It seemed probable that a reduction would be found also in the absence of biologically detectable agent. Mammary carcinoma was too rare in the RIIIf strain without the agent to judge whether ovariectomy would prevent it but adenoma incidence was reduced 15-fold (Pullinger, 1955). Up to and since that time altogether 70 former RIIIf breeders have been ovariectomised without finding a mammary carcinoma subsequently but from the data of Pullinger and Iversen (Table I, 1960) this total is insufficient to judge whether or not a reduction has been achieved.

The C_3Hf substrain bred by Heston and his colleagues has an overall incidence of 22 per cent of mammary carcinoma. Exhaustive biological tests failed to reveal the presence of the mammary tumour agent (Heston *et al.*, 1950; Heston and Deringer, 1952, 1953; Heston, Deringer and Dunn, 1956; Heston, 1958). Through the kindness of Dr. W. E. Heston, progeny of C_3Hf/He mice have been bred in this laboratory for the present experiments. Among 108 breeding females 28 or 25·9 per cent, developed mammary carcinoma (Pullinger and Iversen, 1960). No biological evidence of agent was found (Pullinger, 1960). The fact that after gonadectomy at any age mice of C_3H ancestry, in common with that of some other strains, are liable to develop adrenal cortical hyperplasia and carcinoma, often with biological evidence of secretion of oestrogen (Smith, 1948) might have made it impossible to test the effect on incidence of mammary tumours of lack of oestrogen from known sources unless adrenalectomy had also been done. The risk that actively secreting adrenal cortical tumours would arise in these mice after ovariectomy at puberty proved to be small (Pullinger, 1959). The older the mice when ovariectomised the less this risk is seen to be, though it is never wholly absent in strains with the tendency.

In the present experiments on 50 former C_3Hf breeders a reduction of 7 to 13 fold in mammary carcinoma was observed after late ovariectomy. One mammary carcinoma arose among 50 ovariectomised mothers. This mouse had also a microscopic invasive adrenal cortical carcinoma with evidence in the uterus of oestrogen secretion. One macroscopic non-secreting adrenal tumour occurred.

MATERIALS AND METHODS

The origin, tumour rates and management of the C_3Hf colony and treatment of tissues have been recorded (Pullinger and Iversen, 1960). Animals used in the present experiments were derived from surplus litters of the breeding colony. Females were mated either with their brothers, in which case their precise ancestry was known, or they were collected at approximately the same ages in batches of 6 or 7 and mated at random. The experiment required two groups of breeders, one segregated only, the second segregated and ovariectomised at the same age. Further experiments, to be recorded later but begun concurrently, included substitutions of ovarian hormones after ovariectomy. Whole families of line-bred or batches of randomly mated females were allotted in turn to these several groups. No deliberate selection of mice was made for the various groups apart from the exclusion of a few mothers that had no more than one or two litters. None of the latter developed mammary carcinoma. Because no selection was made, the number of females having the same number of pregnancies are not exactly matched and because breeding was curtailed by segregation or by ovariectomy the most fertile females, presumably capable of bearing 9 to 12 litters, were prevented from having more than 8.

The age chosen for segregation or for ovariectomy determined the actual number of pregnancies of individual mice. Originally this was 8 to 9 months in order that the experiment might start before the first mammary carcinomas were expected. Some have been reported by Heston *et al.* (1950) at 9 months of age but as none had yet been seen in our colony before 12·4 months, segregation and ovariectomy were eventually postponed to 9 to 11 months in all but 3 of the females in Tables I and II.

TABLE I.—*Incidence of Mammary Carcinoma Among Reference and Ovariectomised Former C_3Hf breeders with Number of Litters*

	Reference group		Segregated breeders			
			Intact		Ovariectomised	
Number of litters	Number of mothers	Number with carcinoma	Number of mothers	Number with carcinoma	Number of mothers	Number with carcinoma
1	8	1	2	0	0	0
2	13	1	2	0	0	0
3	9	3	11	1	17	1*
4	16	2	9	1	14	0
5	14	2	6	2	13	0
6	9	4	3	0	3	0
7	4	2	2	0	2	0
8	8	5	1	1	1	0
9	0	0	0	0	0	0
10	5	1	0	0	0	0
11	2	2	0	0	0	0
12	3	1	0	0	0	0
Totals	91	24	36	5	50	1
Per cent		26·3		13·8		2

* Mouse with adrenal cortical carcinoma.

TABLE II.—*Incidence of Mammary Carcinoma in Reference Groups and in Ovariectomised Former C_3Hf breeders with Survival Ages*

Survival age in months*	Reference group		Segregated breeders			
			Intact		Ovariectomised	
	Number of mothers†	Number with carcinoma	Number of mothers	Number with carcinoma	Number of mothers	Number with carcinoma
12	1	1	0	0	0	0
13	0	0	0	0	0	0
14	1	0	0	0	0	0
15	1	1	0	0	0	0
16	1	0	0	0	1	0
17	1	1	0	0	0	0
18	3	3	0	0	1	0
19	3	0	1	0	1	0
20	3	2	2	0	1	0
21	3	1	3	1	2	0
22	3	2	3	0	4	0
23	2	1	2	1	1	1
24	4	3	2	0	1	0
25	6	1	4	0	5	0
26	7	1	2	1	4	0
27	9	2	1	0	6	0
28	10	2	3	0	8	0
29	4	1	4	2	6	0
30	11	2	4	0	3	0
31	4	0	5	0	3	0
32	10	0	0	0	2	0
33	3	0	0	0	1	0
34	1	0	0	0	0	0
Totals	91	24	36	5	50	1
Per cent		26·3		13·8		2

* Age at death or appearance of mammary tumour.
† From Pullinger and Iversen (1960) less 17 which were segregated.

Bilateral ovariectomy was done by the abdominal route using bromethol anaesthesia. The mice were allowed to live until natural death or tumours were found or they were unable to feed. All ovariectomised and segregated mice were seen daily and palpated weekly. After death many adrenal glands and all that were enlarged or nodular were examined microscopically as also were tumours of this and other sites. All 10 nipple regions from samples of mammary glands from both groups were fixed for bulk-staining and for counts of adenomatous nodules.

RESULTS

One mammary carcinoma arose among 50 ovariectomised breeders (Tables I, II and IV). It was a cystic adenoacanthoma in a fourth left nipple region. No trace of ovarian tissue was found. Both adrenal glands were slightly enlarged and nodular. On microscopic examination hyperplasia and widespread infiltration of both capsules and of extracapsular fat by large, vacuolated type B cells of Woolley and Little (1945) were found. Evidence of oestrogen secretion, probably from this latter source, was seen in the uterus, which, at 13 months after ovariectomy, was hypertrophic. The horns were 2·5 mm. and the common uterus 3·0 mm. in

width. The epithelium was of mucous-secreting type with foci of polymorphonuclear cells. Epithelial cells were found in mitosis. In all other females at this age, whether ovariectomised or intact, the remains of genital organs were atrophic. The nipple regions of this mouse were completely involuted apart from the presence of 6 adenomas (included in Table III). A single female with a mammary carcinoma among 50 ovariectomised breeders reveals a reduction in incidence in

TABLE III.—*Incidence of Adenoma in Samples of Reference Groups and in Ovariectomised Former C_3Hf breeders*

	Number of breeders examined	Number with adenoma	Total adenomas	Average number of adenomas per mouse affected
Reference group	37	31	544	17·5
Segregated only	15	12	233	19·4
Ovariectomised	25	11	33	3·0

comparison with 24 females with these tumours among 91 normally bred or 5 among 36 that were segregated after breeding. The degree of this reduction must be judged in relation to parity and survival age of the breeders. Instead of comparing averages, all females that had no more than 8 pregnancies may be considered. Out of 81 normally bred mothers from the reference group taken from Pullinger and Iversen (1940), 20 developed mammary carcinoma and of 36 segregated there were 5 compared with 1 only among the 50 ovariectomised (Table I). The difference in incidence between the reference group and those segregated only is not statistically significant, ($\chi^2 = 1{\cdot}15$). The differences between both segregated and reference groups and those ovariectomised are significant ($\chi^2 = 25{\cdot}4$) and amount to reductions in incidence of 7 and 13 times.

In comparing survival ages, only those animals which lived to at least one month after operation or segregation are included in these results. None of 5 that were ovariectomised and died earlier developed mammary carcinoma. There is no significant difference in survival time between the three groups (Table II and Fig. 1).

Adrenal glands of 28 ovariectomised breeders in addition to the one just referred to were examined microscopically. Results are summarised in Table IV.

TABLE IV.—*Adrenal Cortical Changes and Mammary Tumours in Ovariectomised Former C_3Hf breeders*

Cortical B cells	Number of former breeders	Number with: Adenoma	Number with: Mammary carcinoma
Present only	17	9	0
	4	—	0
Simple hyperplasia	3	0	0
Invasion of capsule	4	2	1
Gross carcinoma	1	0	0

— Indicates no observations made.

Type B, potential oestrogen-secreting cells, were present in the cortices of one or both of 21 pairs ; in 3 pairs they were hyperplastic and in 3 pairs had invaded the capsules but had left no enduring morphological evidence in target organs of secre-

tion of oestrogen. One gross adrenal carcinoma was found at 32 months of age and was not associated with signs of having secreted an oestrogen. No systematic vaginal cornification tests were done.

All nipple areas of 25 ovariectomised breeders were examined by bulk-staining and clearing. Selection of these mice was based on the state of preservation of the cadavers. Thirty-three adenomas (hyperplastic nodules) were found in 11 of these females, a 5-fold reduction compared with intact breeders (Table III).

In former RIIIf breeders a 15-fold reduction of adenomatous nodules was observed after late ovariectomy (Pullinger, 1955). Though mammary carcinoma

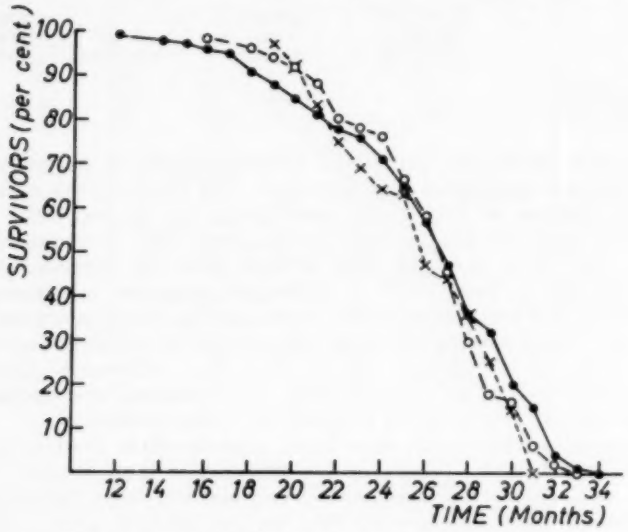

FIG. 1.—Percentage of survivors at ages given in months.

● = The reference group of Pullinger and Iversen (1960) less 17.
× = The group of intact segregated former breeders.
○ = The group of ovariectomised former breeders.

undoubtedly progresses through a stage which, at the present time, is morphologically indistinguishable from adenoma, it is uncertain, in the absence of Bittner's mammary tumour agent, what are the potentialities of most of these adenomas. Thirty-six nodules from RIIIf females lacking evidence of agent failed to grow when grafted subcutaneously or intraperitoneally into young homozygous virgin or breeding females. These hosts lived for further periods of 10 to 18 months thus allowing time and a continued normal hormonal environment to favour their proliferation and change to malignancy. All palpable carcinomas in this strain had proved to be transplantable in subcutaneous tissues of homozygous females and males. The conclusion was reached that the potentialities of the nodules was limited (Pullinger, 1954). However when the mammary tumour agent was present, Browning (1948) grew several small nodules in the anterior chambers of eyes of homologous hosts. Recently, by regrafting nodules from agent-

infected C_3H mice into mammary fat pads from which normal pre-existing mammary epithelium had been excised, De Ome *et al.* (1959) grew mammary carcinoma from nodule transplants that had failed to grow in subcutaneous tissue. The potentialities of agent-free nodules now need retesting by grafting into fat pads by this technique.

Of tumours of other sites in the 50 ovariectomised breeders there were 6 hepatomas, 6 pulmonary adenomas and 1 epithelioma of skin in a mouse which had also a subcutaneous sarcoma. Macroscopic lymphoblastoma and reticulum-celled tumours were found in 4. Polyoma antibody was found in 7 out of 10 of a sample of the C_3Hf colony.

SUMMARY

A reduction in mammary carcinoma occurred after ovariectomy in a group of 50 former breeding females. This varied from 7 to 13 times in comparison with 2 reference groups, one of which was segregated when the mothers were ovariectomised and the second group was bred normally. The incidence of adenomas was reduced 5 times.

The one mammary carcinoma which arose in the group of 50 ovariectomised breeders was associated with an adrenal cortical carcinoma and evidence in the uterus of endogenous oestrogen secretion.

I am indebted to Dr. S. Iversen for Fig. 1.

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CHEMICAL INDUCTION OF MAMMARY CANCER IN PSEUDOPREGNANCY

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In our efforts to elucidate pathways of methylcholanthrene (MCA) action in chemical induction of mammary cancer we have carried out comparative studies on chemical carcinogenesis in several inbred strains of mice. The experimental mice were maintained as normal or forced breeders. Analysis of this data showed few mice that failed to conceive, but developed multiple breast tumours (Ranadive and Hakim, 1957). This accidental observation on high tumour incidence in sterile females attracted our attention. The sterile mice in mating cages were perhaps in continuous state of pseudopregnancy. This suggestion led to further investigation on chemical carcinogenesis in pseudopregnant mice. The present paper is the first report on the subject.

MATERIAL AND METHOD

Three inbred strains of mice—dba(Bar), dba(-MTI) and L(P)—were used for these studies. The breeding stock of the first two strains was imported from Roscoe B. Jackson Memorial Laboratories, Bar Harbor and National Cancer Institute, Bethesda, U.S.A., while L(P) is a line of the strain L(C) from Paris, which was developed in our laboratories and described previously (Ranadive and Hakim, 1958). The line L(P), resistant to spontaneous breast cancer, is found to be particularly susceptible to MCA induced mammary cancer.

Small groups of available young mice of 2–3 months were used for the experiment. Pseudopregnancy was induced in females by two different techniques: (i) by ligation of Fallopian tubes in females and mating these with intact males; and (ii) by mating intact normal females with vasectomised males. The females from the mating cages were kept under daily observation. Frequency of forma-

EXPLANATION OF PLATE

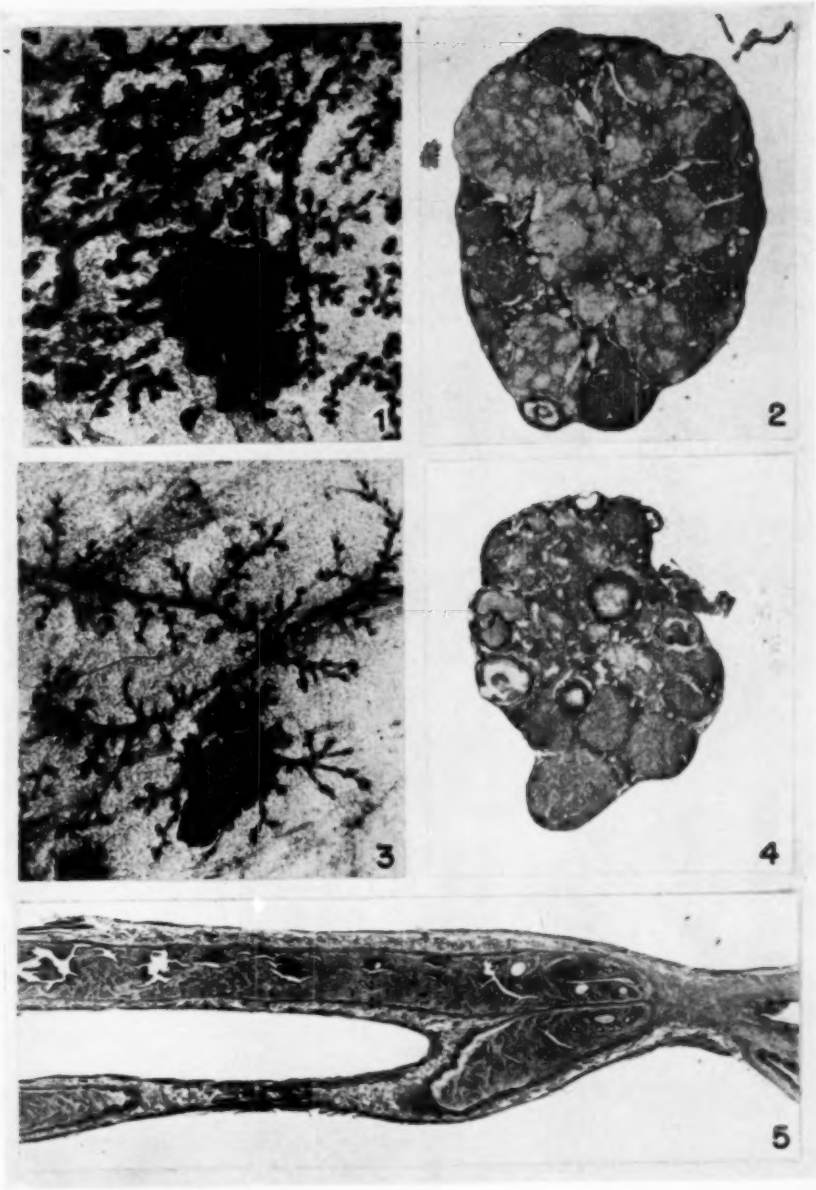
FIG. 1.—Portion of mammary gland whole-mount of an experimental pseudopregnant dba(Bar) female, showing mammary ducts full of acinar-buds and two large focal hyperplastic nodules (βD'). × 19.

FIG. 2.—Section of ovary of an experimental pseudopregnant dba(Bar) female, showing complete leutinisation. A good number of corpora lutea are completely hyalinised. × 24·7.

FIG. 3.—Portion of mammary gland whole-mount of an experimental pseudopregnant L(P) female, showing mammary ducts with few acinar buds and a large localised hyperplastic nodule (αD). × 19.

FIG. 4.—Section of ovary of an experimental pseudopregnant L(P) female with good number of corpora lutea. × 24·7.

FIG. 5.—Section of portion of uterine horns of an experimental pseudopregnant dba(Bar) female, showing thin uterine horns with collapsed endometrial glands and compact lamina propria indicating typical phase of progesterone stimulation. × 12·4.



Ranadive, Hakim, Kharkar.

tion of vaginal plug was noted to ascertain the mating, and daily vaginal smear record was maintained to study the nature of reproductive cycle.

The female mice of both the experimental groups received cutaneous application of solution of 0·25 per cent MCA in thiophene-free benzene twice a week. The tumour incidence data on pseudopregnant mice was evaluated in comparison with that of methylcholanthrene treated virgin and breeder mice of the same strains. The animals were sacrificed when they looked weak and emaciated, either due to a breast lesion or some skin lesions.

Gross mounts of mammary glands were prepared as described earlier (Ranadive, 1945), and stained with haematoxylin. Ovaries and adrenals were weighed in normal saline, fixed in Telly's fixative and paraffin sections were stained with haematoxylin and eosin for routine studies. Pituitaries were fixed in Zenker-formol and stained with modified trichrome P.A.S. method, observations on which will be reported separately.

OBSERVATIONS

(1) *Pseudopregnancy*

Vaginal smears.—Vaginal smear records of all experimental mice were analysed at the end of the experiment and graphs were prepared accordingly.

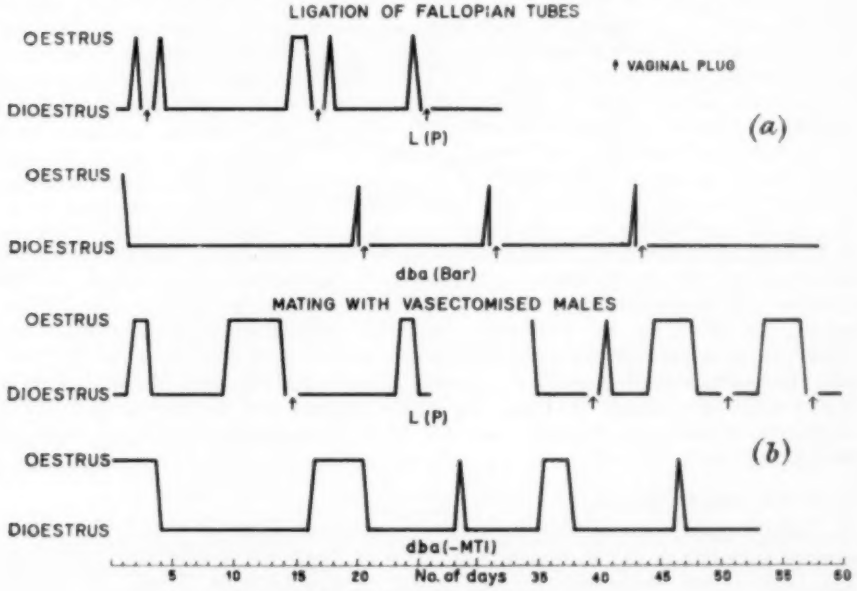


FIG. 6.—Vaginal smear data.

Fig. 6*a* illustrates sex cycle pattern of strain L(P) and dba(Bar) with ligated Fallopian tubes and mated with intact males.

Fig. 6*b* presents vaginal smear pattern of strain L(P) and dba(–MTI) females mated with vasectomised males of the corresponding strain.

The cycle representative of both the experimental groups show characteristic long dioestrus interrupted by a phase of short oestrus. However, there is a noticeable difference in the length of the dioestrus and oestrus in the two groups as also in the occurrence of vaginal plugs. The Fallopian tube ligated group had long dioestrus lasting for 10–12 days and oestrus lasting for only few hours; while the second group had comparatively shorter dioestrus with the oestrus lasting occasionally for 2–4 days. Evidently mice from both the groups must have gone through a state of pseudopregnancy, longer in the first group than in the second.

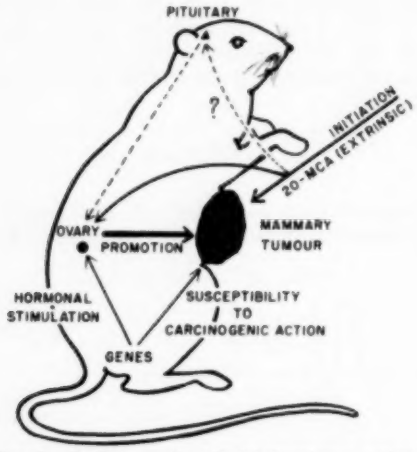


FIG. 7.—Pathways of methylcholanthrene action.

Uterus.—Routine histological study of uterine horns of experimental mice indicated specific progesterone stimulation in more than 60 per cent of the animals. Twenty-four mice out of total 39 had thin uterine horns. The uterine lumen was lined by tall columnar epithelial cells with central nuclei. The lamina propria was compact and endometrial glands were collapsed. Keratinisation of vaginal epithelium was absent. Polymorphonuclear leucocytes were observed in the vaginal cavity. The morphological and physiological findings regarding vaginal and uterine condition were thus consistent with a state of pseudopregnancy (Fig. 5).

(2) *Breast tumour incidence*

Table I gives comparative data on chemical induction of breast tumours in vireins, breeders and pseudopregnant mice of two strains dba(Bar) and L(P). The Table is self explanatory. The groups of treated mice of both the strains were sacrificed between the age of 8–10 months as the animal started becoming emaciated and showing breast or skin lesions. Both in dba(Bar) and L(P) virgins, no palpable tumours were observed in the 8–10 months age group. In breeders of both the strains, a large number of animals (60–65 per cent) developed palpable breast tumours. The breeders developed tumours at age 7–9 months. A noticeable difference in the weights of the ovaries, the number of corpora lutea,

TABLE I.—*Comparative Mammary Tumour Incidence in Virgins, Breeders and Pseudopregnant Mice (Fallopian Tubes Ligation) Treated with 20–Methylcholanthrene*

Strain	Type of mice (Number)	Number of mice with breast tumours	Average age at death (months)	Ovaries		
				Average weight (mg.)	Average number of corpora lutea	Granulosa cell proliferation
Dba(Bar)	V—7	—	9·3	3·6 ± 0·1	4·7 ± 0·4	—
	BR.—16	10/16 (62·5%)	7·9	3·81 ± 0·09	5·34 ± 0·28	6
	Pseud.—8	8/8 (100%)	9·6	14·1 ± 0·3	13·4 ± 2·9	—
L(P)	V—8	—	8·0	1·85 ± 0·25	0·43 ± 0·18	—
	BR.—17	10/17 (60·71%)	8·7	4·49 ± 0·04	4·58 ± 0·13	6
	Pseud.—11	11/11 (100%)	10·1	8·4 ± 1·4	4·69 ± 0·2	9

V = Virgin.
BR. = Breeder.
Pseud. = Pseudopregnant.

and granulosa cells was observed between the virgin and breeder mice treated with the carcinogen. In the strain L(P), the difference was particularly striking as the virgin ovary was very small and weighed hardly 1–2 mg. There were neither well-formed corpora lutea nor granulosa cell lesions. In the breeders many animals showed ovaries which were larger, heavier than normal with well-developed corpora lutea and granulosa cell proliferation. The experimental group of pseudopregnant mice developed palpable multiple breast tumours in every mouse. All the eight dba(Bar) mice and eleven L(P) females developed tumours at age 9–10 months. The ovaries, particularly of dba(Bar) presented a characteristic picture of heavy organs, full of large well-developed corpora lutea (13·4—average number). There was hardly any granulosa cell stromal element present. The L(P) ovary also was comparatively much heavier, double the weight of breeder ovary (8·4 mg.), with well-formed corpora lutea and granulosa cell lesions in many. The pseudopregnant mice which mated, but did not conceive, were virgins for all practical purposes but the difference in their ovaries and breast tumour incidence was striking.

Table II gives breast tumour incidence in the pseudopregnant mice mated with vasectomised males. The breast tumour incidence in pseudopregnant mice of both the strains ranged between 41–45 per cent. It was higher than that in treated virgins but comparatively much less than that in the first batch of pseudopregnant mice with ligated Fallopian tubes.

DISCUSSION

A series of experiments carried out on five inbred strains of mice to study the mechanism of multistep process of chemical carcinogenesis has been described and discussed before (Ranadive and Hakim, 1957, 1958, 1959). Fig. 7 illustrates this data on pathways of methylcholanthrene action.

The carcinogen and/or its metabolites absorbed through the skin and/or circulated through the blood stream to the breast tissue initiates a transformation of normal epithelial cells into malignant cells. The process of cancerous transformation would be accelerated by the continuous action of the carcinogen itself

TABLE II.—*Comparative Mammary Tumour Incidence in Virgins and Pseudopregnant Mice (Mated with Vasectomised Males) Treated with 20–Methylcholanthrene*

Strain	Type of mice (Number)	Number of mice with breast tumours	Average age at death (months)	Ovaries		
				Average weight (mg.)	Average number of corpora lutea	Granulosa cell proliferation
Dba(—MTI)	V—12	—	9·6	4·85±0·15	6·33±0·17	—
	Pseud.—11	5/11 (45·4%)	8·1	7·5±1·6	12·0±0·1	—
L(P)	V—8	—	8·0	1·85±0·25	0·43±0·18	—
	Pseud.—12	5/12 (41·6%)	7·3	2·5±0·2	4·4±1·3	—

V = Virgin.
Pseud. = Pseudopregnant.

which is presumed to be progesterone mimetic as also by adequate hormonal stimulation of the ovary. An indirect pathway of MCA action on the mammary gland through stimulation of luteal function of the ovary was suggested from observations on ovaries of tumour mice. Significant changes were noticed in the ovaries in association with development of chemically induced breast tumours in breeders. The present experiment on pseudopregnant mice was undertaken specially to study the action of excessive luteal stimulation of pseudopregnancy in chemical induction of mammary cancer. Although the groups of pseudopregnant mice studied are rather small, they show a definite increase of tumour incidence over that in virgins.

Andervont and Dunn (1950) have succeeded in inducing tumours in virgins of dba(Bar) and dba(–MTI) with MCA paintings but the incidence has been as low as 30–32 per cent. In our experimental groups we have failed to induce mammary tumours in 10–12-month-old virgins of strains dba(Bar), dba(–MTI) and L(P). In treated breeders of these strains the tumour incidence has been as high as 60–65 per cent (Ranadive and Hakim, 1957), perhaps because of an increase in the luteal factor of pregnancy. In the Fallopian tube ligated pseudopregnant mice the tumour incidence rose to 100 per cent ; all the animals developed multiple breast tumours. This augmentation of tumour incidence may be attributed to continuous progesterone stimulation of pseudopregnancy acting as an adequate promoting factor.

Bonser (1954), while studying chemical induction of mammary cancer in IF strain, intact virgin and ovariectomised mice, first indicated the importance of progesterone stimulation in mammary carcinogenesis. Jull (1954) soon confirmed, from his experiments with ovariectomised IF females, that progesterone in combination with oestrogen acted as an essential promoting agent in carcinogenesis by 20–MCA. He later (Jull, 1957) compared this process of mammary carcinogenesis with skin carcinogenesis promoted by a co-carcinogen like croton-oil. The chemical carcinogen MCA induced the tumour cells. These latent tumour cells grew actively only if the subsequent hormonal stimulation proved to be adequate. Evidently in the present experiment in physiological condition of pseudopregnancy, continuous progesterone stimulus is adequate to stimulate active growth in latent tumour cells of all the MCA treated mice. The findings confirm the importance of continuous progesterone stimulation as an essential promoting factor in mammary carcinogenesis by 20–MCA.

The second group of pseudopregnant females mated with vasectomised males gave tumour incidence as low as only 41–45 per cent. Vaginal smear data indicated little difference in the pattern and frequency of pseudopregnancy. Perhaps the progesterone stimulus under this experimental condition was not adequate for promotion of mammary carcinogenesis. Ligation of Fallopian tubes appeared to be a better method of inducing physiologically functional pseudopregnancy.

During pseudopregnancy, the mammary gland is under continuous excessive stimulation of progesterone. The gland proliferates to develop profuse acinar budding but does not get a chance of normal lactation. According to Marchant's observations (1955) lactation has an inhibitory effect on the development of induced mammary tumours in IF mice. She has lately (1958) confirmed the protective effect of lactation in chemical carcinogenesis of mammary glands. It is perhaps the lack of this protective action of lactation that finally results in higher yield of chemically induced tumours under the physiological condition of pseudopregnancy.

These observations on the importance of hormonal factor of pseudopregnancy as a promoter and accelerator of carcinogenesis might help to explain the higher breast tumour incidence in nulliparous women, as well as in women of certain communities where late marriages and prevention of lactation by not breast-feeding the babies, is in vogue.

SUMMARY AND CONCLUSION

1. Pseudopregnancy has been induced in young female mice of three inbred strains dba(Bar), dba(–MTI) and L(P) by two different techniques :

(*a*) Mating Fallopian tube-ligated females with intact males, and
(*b*) mating normal intact females with vasectomised males.

Virgin, breeder and pseudopregnant mice of three strains have been given cutaneous application of a 0·25 per cent solution of 20-methylcholanthrene in thiophene free benzene twice a week and the incidence of breast tumours has been studied at the age of 8–12 months.

2. None of the experimental virgins, 60–65 per cent of the breeders and 100 per cent of pseudopregnant mice of the first group, developed palpable breast tumours.

The tumour incidence in the second group of pseudopregnant mice was only 40–45 per cent.

3. The high incidence of chemically induced breast tumours in pseudopregnant mice confirms the importance of luteal factor as a promoting agent in chemical carcinogenesis.

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THE DEVELOPMENT OF OVARIAN TUMOURS IN OVARIES GRAFTED FROM MICE PRETREATED WITH DIMETHYLBENZANTHRACENE

Inhibition by the Presence of Normal Ovarian Tissue

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Female mice of the IF strain and its F_1hybrids have been shown to give a high yield of granulosa-celled tumours of the ovary, as well as breast tumours, after skin paintings with an oily solution of 9 : 10-dimethyl-1 : 2-benzanthracene (DMBA) (Howell, Marchant and Orr, 1954). This carcinogen has since been renamed 7, 12-dimethylbenz(α)anthracene.

In a subsequent experiment, which has been preliminarily reported (Marchant, 1959*a*), it was shown that reciprocal bilateral ovarian grafts made between normal mice and mice treated with DMBA resulted in the development of tumours in the majority of mice with ovaries grafted from treated mice, while treated mice bearing grafts of normal ovaries developed only breast tumours.

The present paper reports this experiment in more detail. It also extends it to include the exchange of only one ovary between normal mice and mice treated with DMBA to see whether the presence of normal ovarian tissue would inhibit the development of tumours in ovaries from mice treated with the carcinogen.

MATERIALS AND METHOD

As in the previous experiment, the mice used were young adult virgin female F_1 hybrids derived from $C_{57}Bl$ mothers and IF fathers, and free from mammary tumour agent. Half of them received fortnightly skin paintings of 0·5 per cent DMBA in olive oil, about 1 mg. DMBA being given at each treatment. Six paintings were given in all. After a further 2 or 3 weeks, both ovaries were dissected from the ovarian capsules of the treated mice and from an equal number of untreated mice. The mice were divided into 4 groups, 2 treated and 2 untreated. In the experiment already reported in brief, 2 groups were described in which a bilateral reciprocal exchange of ovaries was made between treated and untreated mice. In the other 2 groups of the present experiment, one ovary of each treated and normal mouse was replaced and the other was reciprocally exchanged. The ovaries were all grafted back inside ovarian capsules by the technique described by Jones and Krohn (1960). The mice were housed 5 to a box and fed on rat cubes known as the Thompson diet (Heygate and Sons) with water *ad lib.* Vaginal smears were done on the mice at intervals. They were kept until they died or their condition made it necessary to kill them. The experiment was terminated 15 months after the grafting operation. At autopsy breast tumours were noted. Ovaries were examined for tumours, then fixed,

sectioned and stained with haematoxylin and eosin. Ovaries not obviously tumourous were serially sectioned and representative sections from different levels examined for early tumours.

RESULTS

The numbers of mice in the 4 groups which came to autopsy, with their survival times and incidences of breast and ovarian tumours, are shown in Table I.

TABLE I.—*Breast and Ovarian Tumours Obtained After Unilateral or Bilateral Exchange of Ovaries Between Carcinogen-Treated and Untreated Mice.*

Hosts	Grafted ovaries	Number autopsied	Number with breast tumours	Number with ovarian tumours			Mean survival (months)
				Macroscopic	Microscopic	Cysts	
Normal	2 treated	18	0	13	1	7	14·4
Normal	1 treated 1 normal	16	0	0	4	13	14·9
Treated	1 treated 1 normal	13	13	0	1	9	3·9
Treated	2 normal	17	16	0	0	4	6·0

1. *Normal hosts bearing bilateral grafts of ovaries from DMBA-treated mice*

The 18 mice in this group survived between 10·3 and 15·3 months (mean 14·4 months) from the grafting operation.

None of the mice developed breast tumours. Near the time of death, several of them had fairly constant oestrus vaginal smears.

Ovarian grafts.—In 14 of the 18 mice solid ovarian tumours were found in one of the grafted ovaries. Thirteen were macroscopic, most of them being 1 cm. or more in diameter. Six of these 14 mice had large cysts filled with clear fluid in the contralateral ovarian grafts. Of the 4 remaining mice, one had bilateral cysts and the other had only atrophied remnants of ovarian tissue.

Histologically, 1 of the 14 tumours was a haemangioma. Five were typical granulosa-celled tumours. Five others were granulosa-celled tumours whose cells showed varying degrees of luteinisation and the remaining 3 were almost completely luteinised.

Oestrus or suboestrus vaginal smears were shown by the majority of the mice with solid ovarian tumours, but not by those without.

2. *Normal hosts bearing grafts of one normal ovary and one ovary from a DMBA-treated mouse*

The 16 mice in this group survived between 14 and 15 months from the grafting operation (mean 14·9) months. The experiment was terminated at 15 months to make this group comparable with the previous group.

None of the mice developed breast tumours. Vaginal smears done near the end of life were very variable, but few showed oestrus activity at that time.

(a) *Grafts of ovaries from DMBA-treated mice.*—In 4 of the 16 mice, tumour nodules were detected only after microscopical examination of the ovaries. One was attached to a small cyst filled with clear fluid and it was a luteinised granulosa-

celled tumour, as was 1 of the other nodules. The remaining 2 very small nodules of granulosa-celled tumour were not luteinised.

A further 7 grafts of ovaries from treated mice had developed into cysts filled with clear fluid, varying in size, but of the order of 1 cm. diameter. Another one had become a fibrinous clot. In the walls of the cysts, the only recognisable ovarian tissues remaining were heavily pigmented lutein cells and, occasionally, hyalinised fragments of corpora lutea. Similar tissue was found in the remnants of the other 4 ovaries grafted from DMBA-mice.

(b) *Grafts of normal ovaries replaced in their hosts.*—None of the normal ovaries replaced in their normal hosts contained tumour nodules.

In 9 of the 16 animals, cysts filled with clear fluid arose, which were often 1 cm. or more in diameter. In their walls, remnants of pigmented or hyalinised lutein tissue were found. Four of these cysts were in mice which also had cysts in their ovaries grafted from DMB-treated mice. The remaining 7 grafts were atrophied and diffusely luteinised and 4 of them contained remnants of hyalinised corpora lutea. In 3 of the grafts a very few small follicles were found.

3. *DMBA-treated hosts bearing grafts of one normal ovary and one ovary from a DMBA-treated mouse*

The 13 mice in this group survived between 1 and 5 months (mean 3·9 months) from the grafting operation. The short survival time was due to the appearance and rapid growth of breast tumours in all 13 mice. Nine of them developed multiple breast tumours, 2 having as many as 5 tumours.

Histological examination of the tumours showed that they were adenocarcinomas, frequently of the papillary cystic type, with abundant fibroblastic stroma and slight secretion. Squamous metaplasia was not frequently seen and sebaceous metaplasia was only seen in one tumour.

Vaginal smears were again very variable, but with little evidence of oestrus activity.

(a) *Grafts of ovaries from DMBA-treated mice replaced in their hosts.*—In 1 of the 13 DMBA-treated mice a nodule of granulosa-celled tumour was found in the host's own replaced ovary. Clear fluid-filled cysts 0·5 to 1·0 cm. diameter with pigmented cells in the cyst walls were found in 6 others. The remaining 6 grafts were very atrophied and were composed of a few pigmented cells.

(b) *Grafts of ovaries from normal mice.*—No tumours developed in grafts of ovaries from normal mice implanted in DMBA-treated hosts. In 5 animals they developed into cysts filled with clear fluid. In the walls of the cysts normal ovarian tissue, composed mainly of follicles and corpora lutea, was found. The remaining 8 grafts of normal ovaries were similarly composed and they showed slight atrophy.

4. *DMBA-treated hosts bearing bilateral grafts of ovaries from normal mice*

The 17 mice in this group survived 2·5 to 11 months (mean 6 months) from the grafting operation. All except the mouse dying after 2·5 months developed breast tumours, 7 of them having multiple tumours.

Histologically the breast tumours were adenocarcinomas, usually with abundant fibroblastic stroma and slight secretion. Foci of squamous metaplasia were occasionally seen.

Vaginal smears showed infrequent periods of oestrus. No tumours were found in these mice.

Ovarian grafts.—In 4 of the 17 mice unilateral cysts were found. They were 0·5 to 1·0 cm. in diameter and filled with clear fluid. Follicles were present in the cysts walls. The other ovarian grafts were somewhat atrophied but normal ovarian structures were present in them, namely follicles, corpora lutea, pigmented cells and, in some of the older grafts, hyaline corpora lutea characteristic of C57Bl/IF hybrids.

DISCUSSION

Of the 18 normal mice of group 1 bearing bilateral ovarian grafts from mice treated with DMBA for 3 months, 14 (78 per cent) developed ovarian tumours, 13 being macroscopic. When treated ovaries were transplanted, a small proportion of them probably contained early tumour nodules. A previous experiment (Marchant, 1959*b*) in which DMBA-treated mice were killed at monthly intervals showed the earliest detectable tumour nodule in 1 of 10 mice killed after 3 months, after which there was a roughly linear increase in the proportion of mice with tumours as time increased. It is certain that many of the 14 tumours in the present experiment must have arisen from ovaries in which no detectable tumour nodules were present at the time of grafting. The environment of the untreated hosts whose own ovaries had been removed allowed the development of such nodules and their growth to a large size.

The untreated mice of group 2 bearing grafts of one normal ovary and one from a DMBA-treated animal survived a similar length of time to those of group 1. However, no macroscopic ovarian tumours developed and microscopic tumour nodules were found in only 4 of the 16 animals (25 per cent) in the ovaries grafted from treated mice. It is considered likely that these tumour nodules were all present at the time of grafting, since a small proportion of animals previously killed after 3 months DMBA treatment had tumours (Marchant, 1959*b*). However, the fact that no macroscopic tumours developed reveals the inhibitory influence of the normal ovaries grafted on the contralateral side.

Group 3 also shows this inhibitory effect. In the 13 DMBA treated animals grafted with one normal ovary and one from a treated animal, only 1 microscopic tumour nodule was found in an ovary from a treated animal. The survival of this group of animals was 3·9 months from grafting and this should have been ample time to yield a high proportion of tumours from the pretreated grafts if no inhibitory factor had been present. The single microscopic tumour found had probably been induced by the 3 months DMBA treatment prior to the time of grafting.

The inhibition by normal ovarian tissue of tumour development in ovaries grafted from mice pretreated with DMBA falls into line with the inhibition of ovarian tumour development by other methods. For instance, after grafting an ovary into the spleen of castrated rat tumours will appear in the grafts, but these are prevented if one ovary is left intact (Biskind and Biskind, 1948). Similarly, the induction of ovarian tumours in mice after X-ray treatment may be prevented by intact ovarian function (Li, Gardner and Kaplan, 1947; Lick, Kirschbaum and Mixer, 1949; Kaplan, 1950). Exogenous oestrogen administration was also found to inhibit ovarian tumourigenesis after intra-splenic grafting (Li and

Gardner, 1949) and after X-rays (Gardner, 1950). However, when stilboestrol dipropionate was administered simultaneously with DMBA, it failed to inhibit the development of ovarian tumours (Marchant, 1957). In this case the hormone was administered along with the carcinogen by skin paintings of a mixture of the two substances in olive oil. This method of administration may have been ineffective because of different rates of assimilation of carcinogen and hormone, the hormone probably being metabolised away more quickly than the carcinogen on account of their differing solubilities.

SUMMARY

First generation hybrid mice from C_{57}Bl mothers and IF fathers were given pretreatment of 6 fortnightly skin paintings of olive oil, each containing 1 mg. of 9 : 10-dimethyl-1 : 2-benzanthracene (DMBA). Both of their ovaries were then reciprocally exchanged with those of similar untreated mice, or one ovary only was exchanged and the other reimplanted. The grafts were made into the ovarian capsules.

Of the 18 untreated mice with bilateral grafts of pretreated ovaries, 14 developed ovarian tumours, 13 being macroscopic. In the 16 untreated mice with unilateral grafts of pretreated ovaries and contralateral normal ovaries, 4 microscopic tumours were found in the grafts of pretreated ovaries. In 13 DMBA-treated mice with similar unilateral grafts, only 1 microscopic ovarian tumour was found in a pretreated ovary. The 17 DMBA-treated mice with bilateral normal ovaries did not develop any ovarian tumours.

It is considered that the presence of the grafts of normal ovaries inhibited the development of ovarian tumours in the mice with unilateral grafts from mice pretreated with DMBA. The small number of microscopic tumours found were probably present at the time of ovarian grafting.

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THE DEVELOPMENT OF OVARIAN TUMOURS IN OVARIES GRAFTED FROM MICE PRETREATED WITH DIMETHYLBENZANTHRACENE

The Effects of the Grafting Operation Itself and the Effects of Grafting Ovaries from Mice at Early Stages in Pretreatment with the Carcinogen

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Skin painting of female mice of the IF strain and its F_1 hybrids with an oily solution of 9 : 10-dimethyl-1 : 2-benzanthracene, now renamed 7, 12-dimethylbenz(α)anthracene, induced breast tumours and granulosa-celled tumours of the ovary in a high proportion of animals (Howell, Marchant and Orr, 1954).

In a subsequent experiment (Marchant, 1959*a*, 1960) ovarian tumours arose in 14 out of 18 normal mice whose own ovaries were removed and replaced with those of mice which had been pretreated with the carcinogen over a period of 90 days. Large cysts filled with clear fluid were also found in 7 of these animals. Another experiment (Marchant, 1959*b*) had shown significant changes in ovaries examined after 90 days of pretreatment with the carcinogen. They were atrophied, with almost complete destruction of follicles and diffuse luteinisation, and early tumour nodules had just begun to make their appearance.

The present experiments were designed to discover whether ovaries grafted in much earlier stages of pretreatment with the carcinogen would develop ovarian tumours and cysts, and to see whether the grafting operation itself was effective in giving rise to tumours or cysts.

MATERIALS AND METHODS

All the mice used in this investigation were young adult virgin females lacking the mammary tumour agent. They were F_1 hybrids derived from $C_{57}Bl$ mothers and IF fathers. They were housed 5 in a box and fed on rat cubes, known as the Thompson diet, with water *ad lib.*

The donor mice were given skin paintings of a 0·5 per cent solution of dimethylbenzanthracene (DMBA) in olive oil once a fortnight, commencing when they were 2 to 3 months old. About 0·2 ml. (containing 1 mg. DMBA) was given at each treatment, distributed over dorsal and ventral surfaces of the body. At periods of 10, 20, 30, 40 or 60 days from the first carcinogen treatment, the ovaries were removed from a group of carcinogen-treated mice and grafted bilaterally to the ovarian capsules of untreated host mice, 2 to 3 months old, whose own ovaries had been removed. Another group of host mice received grafts of ovaries from untreated donors.

Vaginal smears were done on the animals at intervals. They were kept until they died or until their condition made it necessary to kill them. At autopsy

the ovarian grafts were examined for tumour and histological material was taken for microscopical examination.

RESULTS

The results of bilateral grafting of ovariectomised mice with ovaries from DMBA-treated donors are summarised in Table I.

TABLE I.—*Results of Bilateral Grafting of Ovaries from Mice Treated Fortnightly with Dimethylbenzanthracene (DMBA) for Different Periods to Normal Hosts Whose Own Ovaries Were Removed.*

Grafted ovaries (days since 1st DMBA)	Number autopsied	Number with ovarian tumours			Mean survival (months)
		Macroscopic	Microscopic	Cysts	
10	4	1	2	3	14·1
20	7	7***	1*	3	17·8
30	8	8*	1*	2	17·3
40	6	3	0	4	18·7
60	9	7****	1	6	15·8
Untreated	10	0	0	8	19·9

* Bilateral tumours.

Mice with ovaries grafted 10 days after first DMBA-treatment

Four mice in this group were autopsied in a mean time of 14·1 months (range 13½ to 14¼). In one of these animals a pseudofollicular granulosa-celled tumour with a mean diameter of about 2·0 cm. was found in one ovary. In 2 others, nodules of undifferentiated granulosa-celled tumour were found in the walls of large, fluid-filled cysts. In 1 of these mice the contralateral ovary was also cystic ; in the other it was very atrophied and yellow and composed of a few large pigmented cells, as were most non-tumourous ovaries in the experiment.

In the fourth mouse a large cyst filled with clear fluid was found in 1 ovary, but there was no tumour tissue. Vaginal smears of these mice all showed evidence of oestrus activity near the time of death.

Mice with ovaries grafted 20 days after first DMBA-treatment

Seven mice in this group were autopsied after a mean time of 17·8 months (range 13·3 to 21 months). All of the mice in the group developed ovarian tumours varying in size from 0·4 cm. diameter to 1 with 3 lobes, each lobe being over 1 cm. diameter. Two mice had bilateral tumours. Three mice had cysts filled with clear fluid in the contralateral ovary, while in the remainder the second ovary was very atrophied and yellow.

The tumours were granulosa-celled, but showed a variety of histological structure. One small tumour was relatively undifferentiated. The large, three lobed one was partly undifferentiated, partly pseudofollicular and partly cribriform with some luteinisation of the tumour cells. Most of the remainder showed a tendency to a tubular arrangement of cells with varying degrees of luteinisation of them. Four of the 7 mice showed evidence of oestrus or suboestrus activity near the time of death.

Mice with ovaries grafted 30 days after first DMBA treatment

Eight mice came to autopsy in a mean time of 17·3 months (range 13·8 to 20·8). All developed ovarian tumours varying in size from 0·4 cm. to well over 1 cm. diameter. One mouse also had a tiny tumour in the contralateral ovary. Two others had cysts about 1 cm. diameter in the second ovary. Two tumours were solid, undifferentiated granulosa-celled tumours, 3 were pseudofollicular and 2 were very heavily luteinised, the other 2 having the tubular arrangement of cells. Non-tumourous ovaries were atrophied and pigmented. Vaginal smears showed some evidence of oestrus activity, except in the mice with the heavily luteinised tumours.

Mice with ovaries grafted 40 days after first DMBA treatment

The 6 mice in this group survived a mean time of 18·7 months (range 14·5 to 20·8). Three of them developed macroscopic tumours in 1 ovary, 2 having large cysts in the contralateral ovary. A fourth mouse had a large cyst in 1 ovary ; the fifth had 2 cystic ovaries about 0·3 cm. diameter. In the sixth, only atrophied pigmented ovarian remnants were found.

The large tumours were granulosa-celled with luteinisation in the cells of 2 of them, the third being very haemorrhagic. Oestrus activity was detected in 2 of the mice with tumours, but not in the other 4.

Mice with ovaries grafted 60 days after DMBA treatment

Nine mice in this group were autopsied in a mean time of 15·8 months (range 12·5 to 19·5). All had ovarian tumours. Two had bilateral macroscopic tumours and in 1 of these mice 1 of the tumours was attached to a large cyst filled with clear fluid. Two other mice had similar large tumours attached to large cysts in 1 ovary only, the other being atrophied. Four had macroscopic tumours in 1 ovary only and 2 of these had cysts in the other. The last mouse had large bilateral cysts and the wall of 1 contained tissue in which a small tumour was found microscopically.

Two of the tumours in these animals were rather undifferentiated granulosa-celled tumours, with some degree of luteinisation of cells. With the exception of 1, the remainder had the tubular structure already described, with variable luteinisation. The exception was an ovary about 0·4 cm. diameter composed of " corpora albicantia ". Although this ovary was undoubtedly enlarged it was not considered neoplastic and has been excluded from the tumours in the table of results. A similar condition is frequently seen in the ovaries of C57Bl/IF mice after treatment with DMBA (Marchant 1959*b*) and it seems to arise as a result of " hyalinisation " of corpora lutea.

Mice with bilateral grafts of ovaries from untreated mice

The 10 mice in this group survived a mean time of 19·9 months (range 17 to 23) from the ovarian grafting operation. None of them developed ovarian tumours. Five mice had bilateral macroscopic fluid-filled cysts and 3 more had unilateral ones. The remaining ovaries were atrophied and usually composed of diffusely luteinised tissue, often heavily pigmented. Hyaline corpora lutea were found in 3 of them. A follicle was found in 1 ovary of each of the 2 mice

without cystic ovaries. One mouse with bilateral cysts developed a mammary adenocarcinoma after 18 months, the only spontaneous breast tumour found so far in mice of the C57Bl/IF constitution.

The majority of mice in this group did not show any evidence of oestrus activity, as judged by vaginal smears near the time of death.

DISCUSSION

The experiments reported above show that a high proportion of tumours can be obtained from ovaries grafted at much earlier stages in pretreatment of mice with DMBA than the 90 days in the original grafting experiment. Those grafted to normal mice only 10 days after a single skin painting of 1 mg. of DMBA in olive oil were able to proceed to subsequent neoplasia in the majority of animals.

In an investigation of the ovarian changes occurring during treatment of mice with this carcinogen, it was not possible to detect anything other than precocious ageing of the ovaries in the early stages of treatment (Marchant, 1959*b*). There was atrophy of tissue due to rapid loss of follicles, followed by reduction in corpora lutea, with their fusion and disappearance or hyalinisation, and the appearance of pigment. The earliest tumour nodule was detected at the stage in DMBA treatment when follicles and oocytes had almost entirely disappeared and the tumours steadily increased in numbers from then on.

In the experiment reported above, in which normal ovaries were grafted to normal mice whose own ovaries had been removed, histological examination revealed only 2 follicles in 20 ovaries. In most cases the ageing of the ovarian tissue had proceeded to a stage comparable with that reached after several months of DMBA treatment, when a considerable number of tumours or tumour nodules would be found in DMBA-treated mice, but no neoplastic nodules were detected in these grafts of normal ovaries. This suggests that the induction of ovarian tumours in mice treated with DMBA is primarily a result of some phenomonon additional to, or quite apart from, the ageing effects with their consequent changes in hormonal balance which occur after treatment with this carcinogen.

The grafting of normal ovaries did give rise to the development of large fluid-filled cysts in the majority of animals. Further evidence for this was found in another experiment in which normal ovaries were grafted to mice treated with DMBA (Marchant, 1960). Again no trace of solid tumour nodules was found. The appearance of these cysts in a series of ovaries which did not develop any granulosa-celled tumours would appear to exclude them from the same category of growth. They were extremely thin-walled and transparent and, when pierced, they collapsed leaving an amount of tissue no greater than would be found in atrophied non-cystic ovaries. Jones and Krohn (1960) found small fluid-filled cysts in orthotopic ovarian autografts after 25 days or less. They considered them to have arisen from proliferating cells derived from the germinal epithelium.

SUMMARY

First generation hybrid mice from C_{57}Bl mothers and IF fathers were given fortnightly skin paintings of olive oil, each containing 1 mg. of 9 : 10-dimethyl-1 : 2 benzanthracene (DMBA). At intervals of 10, 20, 30, 40 and 60 days from the beginning of treatment, both ovaries were removed from a group of mice and

transplanted to the ovarian capsules of untreated hybrids whose own ovaries had been removed. Another group of untreated hybrids received grafts of ovaries from untreated mice.

A high proportion of mice bearing bilateral grafts of ovaries from DMBA-treated mice developed ovarian tumours, most of them being macroscopic. The proportion of mice bearing tumours in each group did not vary greatly with the duration of pretreatment of the ovarian grafts. No ovarian tumours developed in mice grafted with normal ovaries. Large fluid-filled cysts developed in several mice in each group, including those grafted with normal ovaries.

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A QUANTITATIVE STUDY OF A SERUM PROTEIN ASSOCIATED WITH TISSUE GROWTH. LEVELS FOUND IN RATS UNDER VARIOUS PHYSIOLOGICAL CONDITIONS

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In a previous paper (Darcy, 1957) a study was presented of a rat serum protein whose titre was very greatly increased in the blood serum of tumour-bearing rats and to a lesser extent in the serum of rats which were undergoing regeneration, wound-healing, pregnancy, or in very young rats. It was suggested as a working hypothesis that this substance was directly associated with cell division. It was soluble in sulphosalicyclic acid and moved electrophoretically with the α-globulins. This work was confirmed and extended by Campbell, Kernot and Roitt (1959) in the rat, and supported by Glenn, King and Marable (1959) who worked with human serum.

The present study is a quantitative confirmation and extension of the earlier semi-quantitative results. It is based on an immunological method developed for the measurement of specific proteins in serum (Darcy, 1960*a*). It sets out to provide a base-line of values for this particular protein in the serum of untreated healthy adult rats, for pregnant rats, and for very young rats. In the following paper (Darcy, 1960*b*), values will be given for tumour-bearing rats ; in subsequent papers the behaviour of the protein in rats undergoing regeneration and wound-healing, and its physicochemical properties will be described. Its actual identity has not yet been established but of the known serum α-globulins it will be shown to resemble most closely the protein called " fetuin " by Pedersen (1944*a*, 1947) who found it in foetal calf serum, where it forms 45 per cent of the serum protein. Fisher, Puck and Sato (1958) later showed that fetuin was present, although in much smaller amounts, in human adult serum. Meyers and Deutsch (1955), however, found that fetuin contained at least six components by immunological analysis, whereas the present protein appears to have only one (Darcy, 1957).

In addition to the work on the specific protein, some findings are presented about the total serum proteins of foetal and young rats and their mothers, about which little has been hitherto published.

MATERIALS AND METHODS

Animals.—Healthy albino rats were used except where otherwise stated. They were from a colony originally derived from the Wistar strain ; the colony had been subjected to a degree of inbreeding in the past but were currently propagated by cousin mating. They were remarkable for their large size and robust health. They will be referred to as the C.B. (Chester Beatty) rats. They were fed once per day, between approximately 9.30 a.m. and 10.30 a.m. Three highly

inbred strains were also tested, the Wistar, the August and the Marshall, as well as an F1 cross between the last two.

Bleeding.—Animals were bled from the heart under ether anaesthesia between 10 a.m. and noon. The technique devised for bleeding foetal and newborn rats was a modification of the capillary tube method. The animals were removed from their mothers, wiped clean, the heart exposed and punctured so that it bled into the thoracic cavity; from there it was collected by applying the tip of a capillary tube of large size (2 mm. internal diameter, 9 c.m. in length). The dry end of the tube was sealed in a flame giving a " test-tube " in which the blood clotted and was centrifuged. The tube was then cut just above the clot and the serum fed into the tip of a graduated 0·1 ml. pipette.

Measurement of serum protein.—Total serum proteins were determined by the copper-sulphate method of Phillips, van Slyke, Hamilton, Dole, Emerson and Archibald (1950). It was first compared with the Kjeldahl method and found to be accurate for the rat sera. The Kjeldahl method was used for 17 day foetal serum because of its low protein content. The specific protein was determined by the method referred to above (Darcy, 1960*a*), which is a simple quantitative application of the gel diffusion technique of Ouchterlony (1948). An average error of about ± 15 per cent (95 per cent confidence limits) was obtained for routine titration and this was improved in the later part of the work to about ± 7 per cent. The titres are expressed here in arbitrary units. A unit is defined as the amount of the protein in one milliliter of a 1 per cent solution of the acetone-precipitated proteins of a particular sulphosalicylic acid extract of serum from Walker tumour-bearing male rats.

Two preparations of the protein were used for the production of antisera, the starting material being serum of rats bearing the Walker tumour. One was a sulphosalicylic acid extract of the cancer serum; it contained the protein along with several others. The second was purified preparation obtained by the electro phoretic method of Laurence (1956) at pH5, after preliminary ammonium sulphate precipitation to remove albumin. The yield by this method was small and the reproducibility poor. Nevertheless the sample so obtained gave a virtually monospecific antiserum with which most titrations were done. The antisera prepared with the sulphosalicylic acid—extracted protein showed several precipitate bands in the Ouchterlony plate. The specific band could be easily recognised because it was the one nearest the antiserum depot when the protein extract or serum of Walker tumour bearing rats was run against these antisera.

RESULTS

Normal male rat serum

The blood of healthy male C.B. rats was studied from 5 days before birth (17 day foetus) up to 16 weeks of age and the results are shown in Table I. The rats at 0 day were a few hours old at time of bleeding. All sera were from individual rats except those of the 17 day feotuses where each serum represents the pool of the sera of all the males of one litter. The error of titration of the specific protein for the 17 day foetal sera and for half the 22 day foetal sera was only about ± 7 per cent.

It will be seen that the specific protein reaches its highest level (0·866 units per ml.) at about a week after birth and thereafter drops sharply to a low adult

level of about 0·23 between 8 and 10 weeks after birth. The specific protein is therefore 3½ times as high in the blood serum of the 1 week old rat as in the young adult; its proportion to the other serum proteins is over 7 times higher at 1 week than at 10 weeks.

TABLE I.—*Level of the Specific Protein and Total Protein in the Serum of Male C.B. Rats at Various Ages. The Means, Standard Deviations and Number of Sera (in Parentheses) are Given*

Age of rats	Specific protein (units/ml.)	Total serum protein (g./100 ml.)	Specific protein / Total protein × 100
−5 days	0·263 ± 0·045 (4)	2·43 ± 0·20 (3)	10·8
−1 day	0·725 ± 0·220 (20)	2·52 ± 0·18 (18)	28·8
0 day	0·806 ± 0·129 (22)	2·64 ± 0·20 (25)	30·5
1 week	0·866 ± 0·170 (10)	2·93 ± 0·36 (10)	29·5
2 weeks	0·598 ± 0·291 (10)	3·91 ± 0·45 (10)	15·3
3 weeks	0·522 ± 0·186 (10)	4·58 ± 0·44 (10)	11·4
4 weeks	0·320 ± 0·137 (10)	4·88 ± 0·28 (10)	6·6
6 weeks	0·295 ± 0·067 (10)	5·31 ± 0·32 (10)	5·6
8 weeks	0·245 ± 0·069 (23)	5·89 ± 0·28 (23)	4·2
10 weeks	0·231 ± 0·033 (12)	5·82 ± 0·17 (12)	4·0
12 weeks	0·269 ± 0·040 (12)	5·57 ± 0·26 (12)	4·8
16 weeks	0·231 ± 0·040 (12)	6·25 ± 0·25 (12)	3·7

It is of interest that the specific protein was at a low level in the 17 day foetus, but had increased dramatically in the 22 day old foetus (−1 day). The low level in the 17 day foetus was not merely an absolute one but was also relative to the other serum proteins at the time. In this respect the specific protein differs from fetuin in calf serum, which reaches its highest level in foetal serum (Pedersen, 1944*b*). The two proteins are alike, however, in falling sharply in the weeks after birth.

It is reported below that fasting for about 20 hours may produce an increase in titre of the specific protein. It is possible that the irregularities in the titre curve (e.g. at 12 weeks) are the result of differences in the state of feeding of the rats when bled: some of the rats may have broken their fast sufficiently to lower their titre.

An observation made continually throughout this investigation was that the titres of specific protein for any given age group of rats, when plotted as a frequency distribution, rarely gave a symmetrical curve of error, but rather a skew curve with its long arm towards the higher values. The most probable explanation is that individuals with these exceptionally high titres were carrying some hidden infection or other abnormality which caused the increase.

Comparison of various rat strains

Table II shows the level of the specific protein, together with the total serum protein and mean rat weight, for rats 8 weeks of age. Three inbred lines, the Wistar, the August and the Marshall strains were tested, and an F1 hybrid between two of these.

First a comparison was made between the male and the virgin female of the C.B. stock rat at 8 weeks of age. The female shows a value for the specific protein 83 per cent higher than the male, and the difference, by the *t*-test is highly significant ($P < 0{\cdot}001$). Lest this should have been due to chance selection, the pooled

TABLE II.—*Comparison of the Levels of the Specific Protein in the Serum of Various 8 Week Old Rats. The Means, Standard Deviations and Number of Sera used (in Parentheses) Are Shown*

Rats	Specific protein (units/ml.)	Total serum protein (g./100 ml.)	$\frac{\text{Specific protein}}{\text{Total protein}} \times 100$	Mean rat weight (g.)
C.B. ♂	0·245 ± 0·069 (23)	5·89 ± 0·28 (23)	4·2	233
C.B. ♀	0·449 ± 0·077 (13)	6·21 ± 0·18 (13)	7·25	218
Wistar ♂	0·251 ± 0·055 (9)	5·15 ± 0·30 (9)	4·90	122
August ♂	0·255 ± 0·119 (23)	5·21 ± 0·21 (21)	4·33	117
Marshall ♂	0·231 ± 0·025 (9)	5·41 ± 0·67 (9)	4·27	111
August-Marshall F1 ♂	0·323 ± 0·137 (10)	5·97 ± 0·26 (10)	5·45	121

serum of 48 C.B. females 6½ weeks old was titrated and found to have a value of 0·48 ; this agrees well with the value in the table (0·449) since the younger females would be expected to show a slightly higher value. Later three other pooled sera from C.B. female rats averaging about 8 weeks of age were found to have the values 0·450, 0·442 and 0·404.

The value for the specific protein of the male C.B. rats does not differ significantly from that of any of the other strains. With greater numbers and *ad lib* feeding a significant difference might be established, e.g. between the C.B. and the August strain. The failure to obtain such a difference is due to the variability of the titre from rat to rat within a strain. Nevertheless the mean value does not differ greatly from strain to strain ; none of the 3 inbred strains differs from the C.B. by more than 9 per cent. This is noteworthy in view of the fact that C.B. rats weigh about twice as much as the other three-strains at this age.

The variability is considerable and is in general no less in the strictly inbred lines or the F1 than in the C.B. stock. The high mean value for the F1 (August-Marshall) just failed to reach a significant difference at the 5 per cent level from that of either parent strains. It was noted that the values for the ten F1 fell into two sharply defined groups, one centering around 0·25 and the other around 0·50. Since these animals must be presumed to be alike genetically, it can only be assumed that those with the high values were suffering from some undetected disease or other stress. In this connection it is of interest that one of the Wistar rats which had to be rejected before the experiment because it had a wound on its muzzle and could not therefore be regarded as normal, gave a value for the specific protein of 0·51, i.e. twice the mean value for the other animals and significantly different from it. The total serum proteins for this rat was 4·86 g. per 100 ml., i.e. below the average. It should also be pointed out that the ratio of the specific protein to total serum protein is fairly constant from strain to strain except for the high value in the August-Marshall F1. The C.B. females, of course, stand apart.

The August rats had been bled in three groups : one group of 4 bled in October 1957 gave a mean value of 0·19 ; a group of 10 bled in December 1959 gave a mean value of 0·20 ; but a third group of 9 bled in February 1960 gave a mean value of 0·27. The increase in the third group was mainly accounted for by one high value, and entirely by two. This suggests that there may be a mean value characteristic for a strain but that high values can occasionally occur probably as a result of some disease or stress.

Effect of fasting

August rats.—The effect of fasting on the level of the specific protein is shown in the following experiment. A batch of August rats 12 weeks of age was divided by random selection into two groups, one of 7 and one of 6 rats. They were all bled at the same session and under the usual conditions except that the group of 6 had been fasted for 22 hours, the food which remained in their cages having been removed. The other rats had enough food to last most of the day. The results are shown in Table III. It will be seen that the fasted rats have a level of the specific protein which is about 35 per cent higher than the controls. The difference is significant at $P = 0{\cdot}02$. The differences in total serum protein and body weight were not significant.

TABLE III.—*The Effect of Fasting Upon the Level of Specific Protein in the Serum of 12 Week Old Male August Rats*

	Specific protein (units/ml.) Mean S.D.	Total serum protein (g./100 ml.) Mean S.D.	Rat weight (g.) Mean S.D.	Number of rats
Non-fasted	0·187 ± 0·035	6·52 ± 0·39	155·9 ± 8·1	7
Fasted	0·253 ± 0·049	6·60 ± 0·16	147·2 ± 11·1	6
P	0·02	0·2—0·1	>0·1	

C.B. rats.—The experiment was repeated for the C.B. rats but in a somewhat different form. Twenty-four C.B. males, 8 weeks of age, were separated by random selection into two boxes. Since the normal method of feeding of these animals means that they are fasting for about 20 hours by the time they receive their single allotment of food in the morning, the rats in one box were simply allowed the normal quota of food while the others were given food *ad lib.* All animals were bled the next morning without further feeding; there was still food remaining in the box of the *ad lib* fed rats and also in their stomachs. but none in the stomachs of the fasted rats.

The results are shown in Table IV. The *ad lib* fed rats were significantly heavier than the fasted ones; their serum protein reached a higher (but not statistically significant) total than the fasted ones—an interesting difference from the August rats. The specific protein was 48 per cent higher in the fasted rats, yet this difference was not significant ($P = 0{\cdot}1$). However, there was one extremely high value among the fasted rats, namely 0·945, which can only be accounted for on the basis of some undetected disease or other abnormality. If this value is omitted the mean value of specific protein for the fasted rats drops to 0·245, the standard deviation to 0·055 and the difference between fasted and unfasted becomes significant (at $P = 0{\cdot}05$), although it is now only 19 per cent. It seems reasonable to assume that fasting produces an increase (of about 20 per cent for this degree of fasting) in the specific protein of C.B. rats.

TABLE IV.—*The Effect of Fasting Upon the Level of Specific Protein in the Serum of 8 week old Male C.B. Rats*

	Specific protein (units/ml.) Mean S.D.	Total serum protein (g./100 ml.) Mean S.D.	Rat weight (g.) Mean S.D.	Number of rats
Fed *ad lib*	0·205 ± 0·036	5·96 ± 0·26	263·8 ± 9·3	12
Fasted	0·303 ± 0·209	5·75 ± 0·28	247·7 ± 17·0	12
P	0·1	0·1—0·05	<0·01	

In order to see how quickly the specific protein decreased in the serum after feeding, a batch of 27 male C.B. rats, 9 weeks of age, were tested in the following way. At 9.30 a.m., and before they were fed, 9 of these animals were selected randomly for bleeding. Food was then given to the remainder and $2\frac{1}{2}$ hours later (noon) another randomly selected 9 were bled. The remaining 9 were bled $4\frac{1}{2}$ hours after feeding. The values for the specific protein in the three groups were $0{\cdot}283 \pm 0{\cdot}108$, $0{\cdot}231 \pm 0{\cdot}032$, and $0{\cdot}240 \pm 0{\cdot}038$ respectively. Although there was a drop in the level of about 20 per cent at $2\frac{1}{2}$ hours after feeding, this difference was not significant ($P = 0{\cdot}2$). Nor were there any significant differences in total serum protein.

Effect of pregnancy

The effect of pregnancy on the specific protein and total protein (both determined on the same sera) is shown in Table V. Also included in the table are data from rats which had just littered by a few hours ($22 + 1$ days). It will be seen that the specific protein increases steadily up to the day of delivery (22 days), both absolutely and in relation to the total serum proteins. The total protein, on the other hand, increases for a while and then undergoes a significant drop (about 20 per cent) from the 17th to the 22nd day of pregnancy ($P = 0{\cdot}01$), and it is interesting that the specific protein does not seem to be affected by this. The higher level of specific protein attained after delivery ($22 + 1$ days) than before may be an effect of the birth upon the tissues of the mother. The level reached just before delivery (22 days) is about $2\frac{1}{2}$ times the normal female level.

TABLE V.—*Level of Protein in the Serum of Pregnant C.B. Rats, Showing the Means, Standard Deviations and Number of Sera (in Parentheses) Used*

Days pregnant	Specific proteins (units/ml.)	Total protein (g./100 ml.)	Specific protein / Total protein × 100
0	$0{\cdot}449 \pm 0{\cdot}077$ (13)	$6{\cdot}21 \pm 0{\cdot}18$ (13)	7·2
7	$0{\cdot}632 \pm 0{\cdot}115$ (10)	$6{\cdot}26 \pm 0{\cdot}30$ (10)	10·1
12	$0{\cdot}845 \pm 0{\cdot}251$ (10)	$6{\cdot}42 \pm 0{\cdot}49$ (10)	13·2
17	$0{\cdot}882 \pm 0{\cdot}174$ (10)	$6{\cdot}40 \pm 0{\cdot}40$ (10)	13·8
22	$1{\cdot}053 \pm 0{\cdot}291$ (15)	$5{\cdot}29 \pm 1{\cdot}16$ (15)	19·9
22+1	$1{\cdot}301 \pm 0{\cdot}337$ (10)	$6{\cdot}18 \pm 0{\cdot}41$ (10)	21·1

17 day foetal rats

Table VI summarizes the results of studies on the serum of 17 day C.B. foetuses. Each foetal serum tested was a pool of either the male or female sera of one litter. The male foetus of this age has 37 per cent as much protein in its serum as its mother and only 31 per cent as much specific protein. Both mother and foetus have a low ratio of specific protein to total proteins compared to the later stages of pregnancy. The specific protein of all sera was titrated with an error of about $\pm$ 7 per cent.

22 day foetal rats

Table VII shows the results for 22 day male C.B. foetuses and their mothers. The sera of individual foetuses were tested. The foetal serum has gained somewhat in total protein over the maternal serum since the 17 day stage but there has been a remarkable increase in the specific protein in the foetus, and to a lesser degree in the mother. The foetal serum still has a lower concentration of

the specific protein than the maternal but this protein is now much higher relatively than in the maternal. It would be of interest to know what developmental event is related to this increase.

TABLE VI.—*Level of Protein in the Serum of C.B. Rat Foetuses and Their Mothers on the 17th Day of Pregnancy. Means, Standard Deviations and Number of Sera (in Parentheses) are Shown*

Litter	Specific protein (units/ml.)	Total protein (g./ml.)
A Mother	0·88	Mothers 6·59 ± 0·46 (3)
Foetuses (male)	0·29 (5 pooled)	Male foetal sera 2·43 ± 0·20 (3)
B Mother	0·85	$\frac{\text{Male foetuses}}{\text{Mothers}} = 37\%$
Foetuses (male)	0·26 (3 pooled)	
C Mother	0·94	—
Foetuses (male)	0·304 (5 pooled)	
,, (female)	0·335 (6 pooled)	$\frac{\text{Specific protein}}{\text{Total protein}} \times 100$
D Mother	0·72	Mothers Male foetuses
Foetuses (male)	0·20 (10 pooled)	12·85 10·8
,, (female)	0·17 (6 pooled)	
All mothers	0·848 ± 0·093 (4)	—
All male foetuses	0·263 ± 0·045 (4)	
$\frac{\text{Male foetuses}}{\text{Mothers}} \times 100 = 31\%$		

TABLE VII.—*Level of Protein in the Serum of C.B. Male Rat Foetuses and Their Mothers on the 22nd Day of Pregnancy. Means, Standard Deviations and Number of Sera are Shown*

Litter	Specific protein (units/mil.)	Total serum protein (g./100 ml.)
A Mother	1·08	Mothers 5·67 ± 0·66 (3)
Foetuses	0·548 ± 0·069 (4)	Foetuses 2·52 ± 0·18 (18)
B Mother	1·00	
Foetuses	0·56 ± 0·132 (3)	$\frac{\text{Foetuses}}{\text{Mothers}} \times 100 = 44{\cdot}3\%$
C Mother	1·00	
Foetuses	0·633 ± 0·109 (4)	
D Mother	1·17	
Foetus	0·464	$\frac{\text{Specific protein}}{\text{Total protein}} \times 100$
E Mother	1·13	Mothers Foetuses
Foetuses	0·967 ± 0·085 (8)	19 29
All mothers	1·076 ± 0·076 (5)	
All foetuses	0·730 ± 0·219 (20)	
$\frac{\text{Foetuses}}{\text{Mothers}} \times 100 = 68\%$		

It will be noted that only 3 mothers were used for the total protein calculation. This was because the 18 foetal sera were entirely drawn from the litters of these three (C, D, E). It is of some interest that there appeared to be a rough proportionality between the number of foetuses in the 22 day pregnant mothers and the level of specific protein in their blood. This was not investigated further.

New born rats

Table VIII shows a comparison between the blood proteins of C.B. mothers and their offspring a few hours after birth (0-day old). The individual sera of the young were examined. There has been remarkably little change as a result of birth. The offspring have a lower concentration of the specific protein than

their mothers in all cases and significantly so in three (the mother's value being more than two standard deviations higher than the mean values of their offspring).

TABLE VIII.—*Level of Protein in the Serum of New-born C.B. Rats Compared with Those of Their Mothers at the Same Time. Means, Standard Deviations and Number of Sera Employed (in Parentheses) Are Given*

Litter	Specific protein (units/ml.)	Total serum protein (g./100 ml.)
A Mother	1·30	Mothers 6·18 ± 0·41 (10)
Male offspring	0·803 ± 0·072 (6)	Male offspring 2·64 ± 0·20 (25)
Female offspring	0·826 ± 0·088 (5)	Female offspring 2·53 ± 0·18 (35)
B Mother	1·05	
Male offspring	0·94 ± 0·117 (5)	$\frac{\text{Male offspring}}{\text{Mothers}} \times 100 = 43\%$
C Mother	1·46	
Male offspring	0·835 ± 0·071 (6)	
D Mother	1·16	
Male offspring	0·642 ± 0·063 (5)	$\frac{\text{Specific protein}}{\text{Total protein}} \times 100$
		Mothers 20·1 / Male offspring 30·5
All mothers	1·243 ± 0·177 (4)	
All male offspring	0·806 ± 0·129 (22)	
$\frac{\text{Male offspring}}{\text{Mothers}} \times 100 = 65\%$		

Colostrum and milk were obtained from mothers by the technique of Luckey, Mende and Pleasants (1954), in order to see if the specific protein was present. Sufficient amounts of colostrum for quantitative tests were obtained from two rats. In one the specific protein, though present, was well under 0·2 units/ml., while in the other it was found to contain 0·15 units/ml. (± 7 per cent). A sample of milk obtained 10 days after delivery, from another rat, contained about 0·09 units/ml. It should be mentioned that about $\frac{4}{5}$ths of the 0–day old rats had colostrum in their stomachs at the time of weighing and bleeding.

Certain other points in the table are of interest. The difference in specific protein between the male and female offspring in litter A is not significant (P = 0·3), but the 0–day males in general had a higher total serum protein content than the females (P = 0·05). The 0–day males were also heavier (P = 0·05) than the 0–day females (6·90 ± 0·54 g. compared with 6·65 ± 0·64 g., there being 40 animals in each group).

DISCUSSION

This quantitative study of the rat serum α-globulin, previously found to be associated with tissue growth, has confirmed the earlier semiquantitative results. The protein has been shown to reach a high level in foetal and young rats and in pregnant females, compared with the normal adult level.

The normal adult level is about 0·24 units per ml. of serum for male C.B. rats ; there is a steady decline to this level from a maximum value of about 0·87 at 1 week of age. The growth rate of the rats can be shown to decline in a similar way, the percentage increase in weight per week falling off steadily starting a week or two after birth. The fact that the specific protein levels off at the time when rats are usually considered to be " adult " is itself of interest in connection with the definition of this stage.

Other rat strains tested gave values for 8 week old males closely approxi-

mating that for C.B. males. Since growth rate of the C.B. rats was considerably higher than for these other strains this raises a difficulty for the hypothesis that the specific protein is directly concerned with growth. It may be, however, that the level of the protein in the serum need not be exactly proportional to the growth rate, especially between different rat strains. The results indicated, moreover, that the C.B. rats had a slightly higher level (about 10 per cent) than the slow growing August rats, a difference which might prove significant if large numbers were tested.

A surprising finding was that the 8 week old female C.B. rats showed a level of the protein which was about 80 per cent higher than the males. This is an important clue as to the role of the protein. On the growth hypothesis it could be argued that this reflected the higher growth activity in the female reproductive system. A study of the female during the oestrus cycle would test this idea.

During pregnancy the level of the specific protein in the mothers' blood increased steadily with the growth of the foetuses and at birth reached a level three times as high as in the normal female. It is too early to speculate about the reason for the increase, beyond noting its association with foetal growth. It is of considerable interest that the 20 per cent drop in total serum protein of the mother which occurs between the 17th and 22nd day of pregnancy did not affect the level of specific protein in her blood. This drop, moreover, was not accompanied by a corresponding increase in the total serum protein of the foetus.

Some interesting data were collected on the serum proteins of the foetal rat. The specific protein was low at 17 days, but by 22 days it had increased three-fold, with very little increase in the total serum protein. It would be of great interest to know what developmental event is associated with this increase. It might represent a sudden increase of selective permeability of the placental membranes to the specific protein so that it can enter more freely from the mother's blood, or it might represent the starting up of the foetus' own synthetic machinery for this protein. Birth made remarkably little difference to the foetal serum proteins and it is a matter of speculation whether the specific protein found in the colostrum and milk was absorbed undenatured across the gut of the young rat. The low level of the specific protein in the serum of 17 day foetal rats is another fact which appears to disagree with the hypothesis of a direct association between the protein and growth, for the 17 day foetus must be growing very rapidly indeed. But this need not be a disagreement, for the level in the blood represents an equilibrium between inflow and outflow, and either might be abnormal in the 17 day foetus.

It was stated in the introduction that the specific protein most closely resembles, of all the serum proteins, the group of globulins called " fetuin " which are believed to be characteristic of foetal protein. The evidence on which this is based will be presented in a later paper. But it is supported by the present finding that both are high in the foetus and young animal. However, fetuin reaches its peak in the foetus (or the calf) while the present protein appear to reach its peak at about a week after birth. Furthermore, the present specific protein, unlike fetuin, reaches a high level in pregnant females. These differences, however, may be due to the different species tested.

Fasting plays a significant role in the level of the specific protein. Contrary to what might be expected it causes an increase in the level, this varying from about 20 to 35 per cent for a 20–22 hour fast depending on the rat strain employed.

This fact is another interesting lead as to the role of the protein. It is difficult to reconcile it with the hypothesis of a direct association between the protein and cell division, for fasting is known to cause a decrease in mitotic activity at several sites in the body (Leblond and Walker, 1956). It suggests a relationship between the protein and stress, an hypothesis which could be easily tested.

SUMMARY

1. A study is presented of a rat serum α-globulin which had previously been found to increase considerably during tissue growth, whether normal or neoplastic.

2. The level of the protein in the serum was measured by means of a simple immunological method developed for the purpose.

3. The level of the protein was followed in the male C.B. rat. It was comparatively low in the serum of the 17 day foetus, but increased rapidly thereafter to reach a peak about a week after birth. From there it declined sharply to a low level at about 8 weeks of age.

4. The adult female C.B. rat had a level of this protein nearly twice as high as the male. During pregnancy it increased steadily, reaching nearly $2\frac{1}{2}$ times the normal level just before birth. The protein was also present, though in low concentration, in the colostrum and milk. The relation betwen the foetal and maternal serum protein was studied.

5. The 8 week old males of three inbred lines of rats (Wistar, August, Marshall) had a level of the protein in their serum which differed only slightly from that of the C.B. stock.

6. Fasting caused a significant increase in the protein.

7. The possible role of the protein is discussed.

I would like to express my thanks to Dr. D. J. R. Laurence for valuable assistance in obtaining a purified preparation of the protein.

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A QUANTITATIVE STUDY OF A SERUM PROTEIN ASSOCIATED WITH TISSUE GROWTH. VALUES FOUND IN TUMOUR-BEARING RATS

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In the preceding paper (Darcy, 1960) a study was presented of a rat serum α-globulin, giving its level in the normal animal at various ages, and the increase in this level which occurs during pregnancy and fasting. The main purpose of the present paper is to compare these results with the levels reached during tumour growth. It was hoped to obtain, at the same time, some light on the biological role of this protein which appears so closely related to tissue growth and regeneration (Darcy, 1957).

MATERIALS AND METHODS

Animals.—Male rats were used throughout and, except where otherwise stated, were of the C.B. stock previously described (Darcy, 1960). The inbred August strain rats were used for transplantation of two tumours which arose in that strain. Transplantation was performed by means of a trochar through a 1 cm. incision in the flank which was closed with a single Michel's clip. The C.B. rats used for transplantation were between 6 and 8 weeks of age. Benzpyrene sarcomas were induced by similarly implanting a small pellet of the carcinogen and waiting 4 to 6 months. Butter yellow hepatomas were induced by feeding this substance (4-dimethylaminoazobenzene) as 0·06 per cent of the diet (which contained 20 per cent protein); the tumours appeared in about 8 months. Bleeding was performed under the conditions described in the preceding paper. All operations were carried out using ether anaesthesia.

Measurement of serum protein.—Both the specific α-globulin and the total serum proteins were measured by the methods described in the preceding paper.

RESULTS

Walker tumour

The growth of this tumour in the C.B. rats was extremely rapid and was usually lethal in 3 weeks. Two experiments with this tumour are shown in Fig. 1. In each experiment a batch of 12 C.B. rats of the same age was implanted at one session, and at the indicated intervals thereafter two rats from the batch were bled and their tumours removed and weighed. Two of the 12 rats were kept as controls: in the first experiment they were untreated and bled at 1 and 4 days; in the second experiment they were sham-operated, an empty trochar being inserted, and both were bled at 1 day after the operation.

The first point of interest in the graphs is that the level of the specific protein in the serum of the tumour-bearing rats was already about three and a half times the level of the controls at 24 hours after the operation. Sham-operation of the controls did not appear to have any effect (the normal level for these rats is about 0·24 units/ml.). From 24 hours onwards the specific protein increased in the tumour-bearing rats, although at a reduced rate. The rate increased again starting at about 8 days after transplantation, by which time the tumours weighed between 15 and 36 g. The level of specific protein reached at 12 days after transplantation was 21 times that of the controls in Experiment 1 and 14 times that of the controls in Experiment 2.

While there is a general correspondence between the size of the tumour and the level of the specific protein in the blood, it is not a close one. This can be seen from the shape of the two curves in Experiment 1, and from a comparison

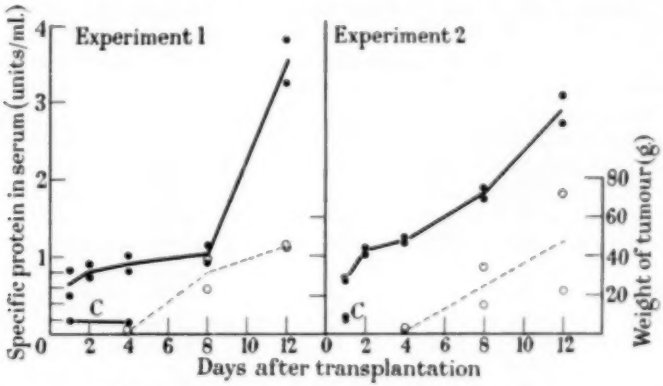

FIG. 1.—Increase of the specific protein in the serum of C.B. rats after implantation of the Walker tumour. C, controls. ●, specific protein. ○, weight of tumour.

of the 8 and 12 day values in Experiment 2. Whatever the basic cause of the increase of specific protein, it is unlikely to be the actual weight of the tumour in the animal: a more likely candidate is the weight of viable tumour cells or, more probably, of actively dividing cells, for the larger Walker tumours had a massive necrosis of their centres and only a cortex of viable cells.

It is instructive to compare the curves for the two experiments. Thus, while the tumours grew somewhat faster up to 8 days in Experiment 1, the level of the specific protein remained considerably lower at 8 days than in Experiment 2. From 8 to 12 days there was a violent upsurge of the protein in Experiment 1 accompanied by a tumour growth that was, if anything, slower than in Experiment 2. This suggests that there may be some sort of partial antagonism between the two quantities, for example the possibility that the tumour is using up the protein so that when tumour growth exceeds a certain rate the outflow of the protein from the blood begins to overtake the inflow.

Specific protein and total serum protein determinations were made on a group of 7 rats with large Walker tumours transplanted 10 to 14 days previously. The mean value for the specific protein was 3·24 units/ml., which agrees well with the

values in the above experiments. The mean value for total serum protein was 4·64 g./100 ml. (as compared with a value of about 5·8 for healthy animals, cf. preceding paper). But the most striking value was the ratio of specific/total protein (×100). This was 69·9, compared with the normal value of about 4·0. The highest value encountered in any normal animal was 30·5 in the new-born rat.

Ascites Walker tumour

The Walker tumour can be made to grow successfully in an ascites form. It adheres mainly to the omentum and mesenteries of the peritoneal cavity where it forms clusters of tumour nodules. But tumour cells also float freely in the ascitic fluid. Table I shows the results of testing the serum and the supernatant ascitic

TABLE I.—*Levels of the Specific Protein and Total Protein in the Ascitic Fluid and Serum of Male C.B. Rats Bearing the Walker Ascites Tumour for 7 Days*

Rat	Specific protein (units/ml.)			Total protein (g./100 ml.)		
	Serum	Ascites	$\frac{\text{Ascites}}{\text{Serum}} \times 100$	Serum	Ascites	$\frac{\text{Ascites}}{\text{Serum}} \times 100$
1	0·90	0·57	63	3·65	2·95	72
2	0·35	0·23	66	2·95	2·60	88
3	0·34	0·22	65	2·95	2·60	88
4	0·55	0·32	58	3·65	2·95	81
5	0·69	0·41	59	3·65	2·95	81
6	0·42	0·26	62	4·16	2·95	71
7	1·08	0·70	65	3·65	3·30	90
8	0·77	0·20	26	6·07	5·72	94
9	1·14	0·48	42	4·33	2·95	68
10	1·61	1·05	65	4·16	3·65	88
11	1·33	0·67	50	4·33	3·12	72
Average	0·835	0·465	56%	3·96	3·25	81%

fluid for the specific protein. The rats (which were 8 weeks old) had been inoculated intraperitoneally with the tumour cells 7 days before. It should be noted that the average value of the specific protein in the serum of normal 8 week old male C.B. rats is about 0·24 (cf. preceding paper). It will be seen that this value has been more than trebled, on the average, in the serum of rats bearing ascites tumours. But in the ascitic fluid itself it has only been about doubled. The level in the fluid varies from 26 to 66 per cent of that in the serum of the same rat.

This observation has an important bearing on the site of origin of the specific protein. It strongly suggests that the protein is not produced by the tumour (which might be expected to secrete it into the ascites) but at some distant site in the body whence it is carried by the blood. This is supported by the fact that the ascites was extremely bloody (it was indistinguishable in colour from whole blood) and that the ascitic supernatant contained on the average 81 per cent as much protein as the serum. But since the specific protein reached a level in the ascitic fluid which was only 56 per cent that of the serum, this suggests the possibility that the specific protein is being selectively withdrawn from the fluid by the tumour cells. When the ascitic supernatant was tested on the Ouchterlony diffusion plate it appeared to contain the same complement of proteins as the serum of the same animal.

The low total protein content of the serum of these rats is noteworthy, being only 3·96 g./100 ml. compared with 4·64 for the 12–14 day old solid Walker tumours. The ratio of specific protein to total protein ($\times 100$) is also low compared with that for the solid tumours, being an average of 21·0 for the serum and 14·3 for the ascitic fluid. Nevertheless it is remarkable that at a time when the total serum protein is so seriously depressed the specific protein should have increased so much above the normal level. The actual weight of tumour in these ascites-carrying rats was small, not more than 10 g. at the most ; but there was also little or no necrosis.

Analysis of the jelly surrounding the Walker tumour

It has been suggested that an increase in the serum glycoproteins may result from a depolymerization of the ground substance of the connective tissue, giving rise to smaller soluble proteins which leak out into the blood (Catchpole, 1950). This hypothesis might reveal the site of origin of the present protein, so it was tested in the following way.

The Walker tumour growing subcutaneously in the C.B. rats produces a considerable quantity of a clear watery jelly in the connective tissue around itself. When this material is excised and centrifuged it yields a white sediment (connective tissue) and a clear supernatant. The supernatant was analyzed for specific protein, total protein, and also for the number of individual serum proteins it contained by means of the Ouchterlony gel diffusion test. This last test, employing rabbit antiserum against serum of Walker tumour-bearing rats, showed that the supernatant contained the proteins of serum, and in approximately the same proportion to one another, with the exception of certain higher molecular weight proteins which appeared to be in relatively lower concentration than in the serum. These larger proteins could be detected by the convex curvature of their lines (Korngold and Van Leeuwen, 1957). Such an effect might be predicted since the proteins presumably get into the jelly by diffusion and there may even be some filtration. Apart from this difference the jelly supernatant appeared to be a dilute form of serum.

TABLE II.—*The Protein Content of the Liquid Phase of the Jelly Surrounding the Walker Tumour Compared with that of Serum*

Rat	Specific protein (units/ml.)			Total protein (g./100 ml.)			Tumour	
	Jelly	Serum	$\frac{\text{Jelly}}{\text{Serum}} \times 100$	Jelly	Serum	$\frac{\text{Jelly}}{\text{Serum}} \times 100$	Size	Condition
1	2·76	4·40	63	2·60	5·60	46	—	—
2	2·64	4·16	63	2·95	4·33	68	—	—
3	0·19	0·30	63	2·60	4·16	63	Small	—
4	0·29	0·78	37	2·60	4·33	60	,,	Good.
5	0·63	1·42	44	2·77	5·21	53	,,	Necrotic.
6	1·67	3·59	47	3·65	5·90	62	,	—
7	0·60	1·84	33	2·77	2·60	106	Large	Mainly good.
8	1·95	4·02	49	2·43	4·51	54	Huge	Part good, part necrotic.
9	2·45	4·14	59	2·95	5·03	59	,,	Necrotic.
Average	1·46	2·74	53%	2·81	4·63	61%		

Table II shows that the jelly contained on the average only 53 per cent as much of the specific protein as the serum, and 61 per cent as much total protein. It is unlikely therefore that the jelly is the site of origin of the increased specific protein in the serum. There is again a case for the argument that specific protein is being selectively taken up from the jelly by the tumour. Certainly the specific protein was lowest relative to the other proteins in the jelly of the two tumours (rats 4 and 7) which showed least necrosis and were in best condition. No explanation can be offered for the case in which the specific protein was in higher relative concentration in the jelly than in the serum (rat 1). In the case where the jelly fluid appears to have a higher protein content than the serum (rat 7), the difference is probably not significant and the extraordinarily low protein content of the serum (2.60 g./100 ml.) may reflect the advanced state of the tumour growth in this animal.

The August tumour PWA.2

This is a transplanted tumour which originated as a mammary carcinoma in the inbred August rats in which it gives 100 per cent "takes". Its interest for the present work is that, in contrast to the Walker tumour, it is very slow-growing and shows little or no necrosis. Ten August males were implanted with

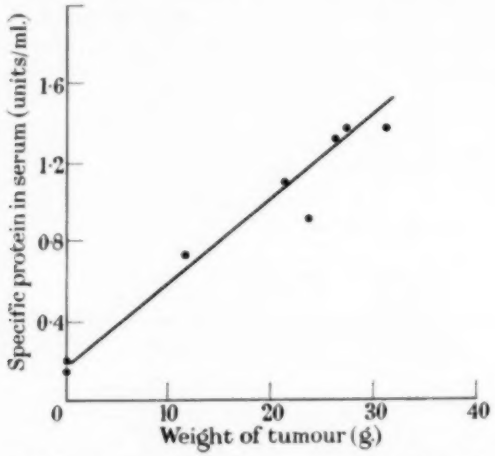

FIG. 2.—Increase of the specific protein in the serum of August rats at 16 days (no tumour) and 73 days after implantation of the PWA. 2 carcinoma.

the tumour and bled as follows: two at 16 days after grafting, two at 37 days, and the remaining six at 73 days after grafting. The results are shown in Fig. 2.

At 16 days after grafting there was no measurable tumour and no increase in level of the specific protein above the normal for these rats (about 0·22 units/ml.; Darcy, 1960). At 37 days after grafting the two animals bled gave titres of 0·56 and 0·29 for the protein. Unfortunately their tumours were not weighed but measured (the length and breadth measured through the skin). They are not

therefore shown on the graph. But when all the tumour sizes were expressed as the product of length × breadth, the two 37 day points straddled the line of regression just below the smallest of the 73 day tumours and did not disturb its slope.

The results show that there is a close relationship between tumour size and level of specific protein, much closer than was shown by the Walker tumour. These August tumours had only a small amount of necrotic-looking tissue in their centres while the Walker tumours were widely necrotic except for a cortex. This suggests that the level of the specific protein is proportional to the mass of living tumour tissue and supports the concept that the basic relationship is between the protein and the mass of actively dividing cells.

It could be objected against this interpretation of the results that the increase of the specific protein in the serum is simply a function of time after inoculation and may not be a function of the actual size of the tumour. To test this point the coefficient of correlation was determined for the 6 sera and tumours which were taken on the 73rd day after transplantation. There was found to be a significantly positive correlation between the level of the specific protein and the tumour weight ($r = 0{\cdot}9073$, $P = 0{\cdot}02 - 0{\cdot}01$), showing that the relationship does not depend on the time of residence of the tumour.

The total serum proteins for the rats bled at 16, 37 and 73 days after tumour inoculation averaged 5·72, 5·72 and 5·17 g./100 ml. respectively and the ratios of specific to total protein (× 100) were 3·15, 7·43 and 21·4.

The August osteosarcoma D.177

This is another tumour which arose in and is transplanted in the August rats. It was studied partly in order to have a sarcoma to compare with the PWA.2, and partly because it is exceedingly fast-growing compared with the PWA.2. Eleven August rats 7 weeks of age were implanted with the tumour and its growth was followed by measuring the length and breadth with calipers through the skin. By ten days after transplantation the mean size of the tumours was 0·93 cm.^{2} (product of the two measurements). On the 15th, 16th and 17th days after transplantation the mean sizes were 5·95, 7·0 and 8·8 cm.^{2} respectively. The animals were bled and the tumours weighed on the 17th day.

The results are shown in Fig. 3. They are completely contrary to what was expected on the basis of previous experience, and appear at first sight to refute the hypothesis that the specific protein is related to tissue growth. Since the osteosarcoma is so much faster-growing than the mammary carcinoma PWA.2, it might be expected to cause a much higher level of the specific protein in the serum. Instead the level (the average is 0·365 unit/ml.) is much lower than that for the PWA.2 tumours at 73 days (where the average was 1·11 units/ml.) and is only about 60 per cent higher than the level found in normal August male rats (0·225 units/ml.).

Furthermore, unlike the other tumours tested, there is no positive relationship between the size of the tumour and the serum level of the specific protein. Indeed there is a slight tendency in the opposite direction, i.e., the larger the tumour the lower the level of specific protein seems to be. This negative relationship was not significant on the present sample, however, ($r = -0{\cdot}235$, $P = 0{\cdot}5$), although it was slightly improved when the estimated necrotic fraction of each tumour was first subtracted from its weight ($r = -0{\cdot}288$, $P = 0{\cdot}4 - 0{\cdot}3$).

These results cast doubt on the hypothesis that the level of specific protein in the serum is always positively related to the total amount of tissue growth going on in the body at a particular time. They do not, however, refute the hypothesis that the specific protein is itself concerned in tissue growth, for it is possible that the rate of growth of the osteosarcoma is such that the specific protein in the serum is removed as fast as it enters, so that only a relatively low level can be maintained.

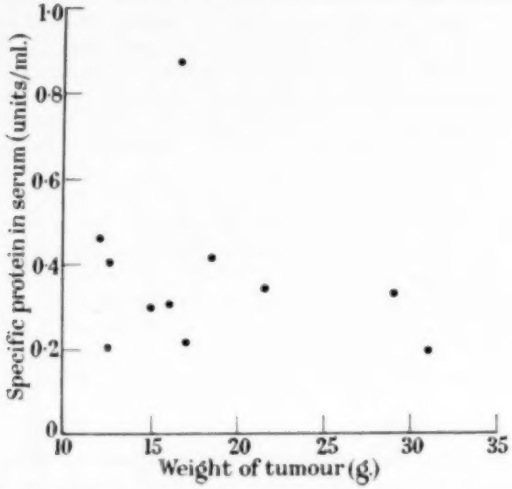

FIG. 3.—Specific protein in the serum of August rats 17 days after implantation of the D.177 osteosarcoma.

Furthermore it is known that August rats have a low capacity for protein synthesis and growth compared with C.B. rats (Elson, 1958).

TABLE III.—*The Level of Protein in the Serum of August Rats Bearing the D.177 Osteosarcoma for 12 and 15 Days. Means and Standard Deviations Shown*

Days after transplantation	Rats	Tumour weight (g.)	Total serum protein (g./100 ml.)	Specific protein (units /100 ml.)
12	5	7·98 ± 3·77	6·00 ± 0·26	0·401 ± 0·116
15	5	22·60 ± 4·55	4·40 ± 0·16	0·653 ± 0·057
P	—	<0·001	<0·001	0·01–0·001

To examine this question further, a batch of ten rats with osteosarcomas were examined, five being selected randomly and bled at 12 days after transplantation, the remaining five being bled at 15 days after transplantation. The results are shown in Table III. The tumour grew faster than in the previous experiment; it had approximately trebled its weight during the 3 day interval. The levels of specific protein in the serum were higher than in the previous experiment, but were still lower than might be expected from the size and growth rate of the tumour. An important observation is that the level was higher at the later stage

of tumour growth, suggesting that there was, in this experiment, a positive relationship between tumour size and level of specific protein.

The effect of necrosis could be examined in this tumour, where its extent was estimated and found to vary from 0 to about $\frac{3}{4}$ of the tumour volume. No relationship was found between the estimated weight of necrotic material and the level of the specific protein in the serum. No relationship was found between tumour size and the extent of necrosis.

Induced hepatomas

These tumours were induced in the C.B. rats by feeding them the azo dye, butter yellow, in a 20 per cent protein diet. Tumours appeared after about 8 months and, where necessary, were confirmed histologically as being hepatomas. The results of testing the sera are shown in Table IV where they are grouped according to the approximate size of the tumours.

TABLE IV.—*The Level of the Specific Protein in the Serum of Rats Bearing Hepatomas Induced by Means of Butter Yellow Feeding*

Rat	Tumour		Specific protein (g./100 ml.)
	Size	Condition	Metastases	
1	No tumour	—	None	0·25
2	Small	Good ; liver normal	,,	0·41
3	,,	Good	,,	0·36
4	Moderate	,,	—	1·2
5	Large	Partly necrotic	—	2·4
6	,,	Good. Liver abnormal	—	1·0
7	Very large	Cystic and a little necrotic. Much ascites	—	1·5
8	,, ,,	Half necrotic. Ascites	—	2·6
9	,, ,,	Mainly good	—	2·6
10	,, ,,	Good	—	3·1
11	,, ,,	Good. 40 g.	None	2·5
12	,, ,,	Partly cystic and haemorrhagic, 15 g., good	Some	1·5
13	,, ,,	Necrotic. Good metastases	Much	2·8
14	,, ,,	Partly cystic	,,	4·7
15	,, ,,	Cystic and necrotic, but metastases good	Very much (40 g.)	7·6
16	,, ,,	Good	Very much (30 g.)	3·8

The first rat in the table had no tumour after 10½ months of butter yellow feeding. Its serum level of specific protein was normal. There is a general correspondence between the size of the tumours and the level of specific protein. The highest titres were found in rats with metastases. Where there is no entry under " metastases " in the table they were either absent or very slight. Rat 15 gave the highest titre ever recorded in this laboratory ; its primary tumour was cystic and necrotic but the metastases were vast ; it had been fed the dye for only 7 months. One animal which was excluded from the table because its tumour was not a hepatoma but a fibrotic tumour, apparently developed from a cholangioma, gave the high titre of 4·2.

These results are comparable with those obtained with the transplanted Walker tumour in C.B. rats. However the possibility must be kept in mind in interpreting them that they may be complicated by liver damage, for there is a possibility

(as results to be presented in a later paper will show) that the specific protein is produced in the liver. This might explain, for example, why the specific protein failed to rise with small tumours (rats 2 and 3) to the extent it did with small Walker tumours, but difference in growth rate of the tumours could also account for this.

The specific protein during carcinogenesis

The following experiment shows the effect of another type of induced tumour, this time a sarcoma, upon the level of the specific protein in the serum, and also the effect during the genesis of these tumours. C.B. male rats were implanted subcutaneously with small pellets of 3 : 4-benzpyrene. Sarcomas usually arise in about 75 per cent of these animals at the site of implantation (and usually surrounding the pellet, which is not absorbed). Seven such rats were bled from the heart at monthly intervals, and the serum level of the specific protein measured. The results are shown in Table V.

TABLE V.—*The Effect of Carcinogenesis on the Specific Protein. Serum Titres of the Protein in 7 Rats at Monthly Intervals Starting Three Months After They Had Been Implanted Subcutaneously with a 3 : 4-Benzpyrene Pellet. The First Appearance of the Tumour is Indicated by the Titre in Bold Type and the Approximate Tumour size (in cm.²) in parentheses*

	Specific protein (units/ml.) in serum				
Rat	3 months	4 months	5 months	6 months	7 months
1	0·18	0·21	0·21	0·22	**3·35** (*25·8*)
2	0·21	0·43	**0·42** (*3·2*)	3·84 (*20·4*)	—
3	0·29	0·40	0·35	0·31	**2·84** (*56·5*)
4	0·44	0·30	0·27	0·25	0·32
5	0·78	0·30	0·47	0·43	**0·44** (*1·0*)
6	0·47	0·40	0·40	0·25	—
7	0·44	0·40	**3·60** (*26*)	—	—

Two of the rats (4 and 6) did not develop tumours during the period under observation. Their serum level of the specific protein remained well within the normal range (0·245, S.D. ± 0·069 ; cf. Darcy, 1960). Of the remaining five rats, two (2 and 7) had tumours when examined 5 months after implantation of the carcinogen. For rat 2 the tumour was still rather small (3·2 cm.²) and the specific protein low (0·42), while in rat 7 the tumour was already large (26 cm.²) and the specific protein high (3·60). At six months the tumour in rat 2 had grown considerably (20·4 cm.²) and the specific protein had increased correspondingly (to 3·84). By 7 months the three remaining rats had tumours ; in rat 5 the tumour was small (1 cm.²) and its specific protein only 0·44, whereas in rats 1 and 3 the tumours were 25·8 and 56·5 cm.² respectively and the specific protein 3·35 and 2·84 respectively.

Two main conclusions can be drawn from these results. The first is that tumours of small but quite important size, in relation to the host's size, can exist in the body without causing the level of specific protein in the serum to rise appreciably. In short, for this particular host-tumour situation the level of specific protein in the serum would be useless as a diagnostic tool. It is interesting to compare it with the Walker tumour in the same animals, where the titre increases 3 to 4-fold

within a day of transplantation. The difference may lie in the different speed of growth of the two kinds of tumour, although this may not be the only cause.

In the period before tumours appeared the level of the specific protein in the serum was higher than in normal rats. For example, at 3 months after implantation the average was 0·40 units/ml. compared with 0·245 in the normal rat. It would be rash, however, to ascribe this to the carcinogenic process, for such an increase might be produced by many stresses, e.g. slight overcrowding in the cages, mild infections, the heart puncture, etc. Furthermore, rat 1, seems to have developed its tumour without any such preliminary increase.

The second conclusion is that the specific protein in the serum increases greatly with a second type of induced tumour, this time a sarcoma. Furthermore, the increase is again roughly in proportion to the size of the tumour, although the fit is by no means exact. An exact fit would not be expected because we are here dealing, not with a single homogeneous tumour like the August PWA.2, but with a group of independent sarcomas each with its own growth rate and other characteristics. The mean size of the 4 large tumours in the table was 32·2 cm.^{2} Doubling this quantity gives the approximate weight in grams. The mean specific protein level of these four tumours was 3·41. This result is comparable with that for the Walker tumour.

The total serum proteins were determined for the blood of other rats bearing large benzpyrene sarcomas, averaging about 40 cm.^{2} The average value was 5·19 g./100 ml. which is relatively high compared with that for advanced Walker tumours (4·64). The value in normal rats is about 5·8. The ratio of specific protein to total protein was variable, for example in three rats whose tumours measured 15·4, 19·5 and 20·7 cm.^{2}, the specific protein was 0·90, 1·01 and 4·26 respectively and the ratios of specific to total protein ($\times 100$) were 17·3, 19·4 and 66·4 respectively.

The specific protein and the " K " lines

In a comparison of cancer serum with normal serum in the rat (Darcy, 1955) using the Ouchterlony gel diffusion technique, it was reported that the cancer sera could be distinguished by the presence of precipitate bands which migrated ahead of the albumin band. These bands were called " K " lines. They were best seen when the antiserum was against normal rat serum. In the present study it was found that one of these bands was caused by the specific protein ; furthermore it was the most prominent one and the one given by most antisera. The K line phenomenon in this case is easy to interpret : the higher concentration of the specific protein in the cancer serum causes its band to move from its normal position in mid-spectrum to take up a position ahead of the albumin band which is usually the leading one for normal serum. The question of whether the change in the molecular size of the specific protein could also play a part will be examined in a later paper.

Discussion

The results of this investigation confirm the earlier semi-quantitative finding of a sharp increase in the serum of tumour-bearing rats of the specific α-globulin under study. The normal adult level of this protein was previously found to be about 0·24 for C.B. male rats (all tumour-bearing rats used were males) and the

highest level encountered for normal males was 0·87 at 1 week of age ; the highest level in females was about 1·2 in the last stages of pregnancy. With the growth of a tumour levels of between 3·0 and 4·0 were common, not only for transplanted tumours but for induced ones. Whatever its role, this protein certainly assumes an important position among the serum proteins during tissue growth. It was demonstrated, in the favourable case of the PWA.2 tumour, that there was a significant positive correlation between the size of the tumour and the level of this protein. This relationship was somewhat masked in the case of the necrotic Walker tumour, strongly suggesting that it is the amount of healthy tumour tissue with which the protein is correlated. But the basic relationship may be between the protein and the total amount of growth in the tumour and in the body (i.e. the mass of growing tissue times its rate of growth). For the only factor common to the various situations in which an increase in the serum level of the protein has so far been observed appears to be growth. There are, however, one or two situations which cannot easily be fitted into this pattern, especially the small but significant increase in the serum level of the protein which occurs during fasting. Fasting is known to inhibit mitosis in several sites of the body. It may be, however, that it increases mitosis in another part of the body to the extent which gives a small net increase over the normal total level. In any case a correlation between the serum level of the protein and growth could not always be hoped for since many other factors may influence the level. The case of the fast-growing osteosarcoma D.177 in the August strain rats may be an example. This tumour caused a relatively small increase of the specific protein in the serum (contrary to what the growth hypothesis would predict) and there was no positive correlation between tumour size and the protein titre, at any rate in the first experiment. This suggested the supplementary hypothesis that rate of withdrawal of the protein from the blood was so great, under the influence of this tumour, that the inflow could only maintain a rather low blood level ; in support of this is the fact that August rats are known to be relatively weak protein synthesizers. An alternative explanation is that the D.177 tumour has some metabolic peculiarity. It would appear, on the whole, that the hypothesis that this protein is directly concerned with tissue growth, remains tenable even though the serum titre of the protein may not always be proportional to growth. It may be, however, that certain conditions will be found (e.g. infections and certain stresses) whose effect on the protein will render the growth hypothesis untenable.

Another hypothesis, namely, that the specific protein originates by depolymerization of the ground substance of the connective tissue at the site of tissue growth, was put to the test by analysing the watery jelly in the connective tissue surrounding the Walker tumour. On the hypothesis, a higher concentration of the protein would be expected in the jelly than in the serum. Instead it was found that the aqueous phase of this jelly contained only 53 per cent as much specific protein as the serum on the average. Another blow for this hypothesis is the finding that when the Walker tumour is grown in the peritoneal cavity, the ascitic fluid contains only 56 per cent as much specific protein as the serum on the average.

In both the above experimental situations, the jelly fluid and ascitic fluid were found to contain all or nearly all the proteins of serum, though in lower concentration. The ascitic fluid was actually bloody, though it did not clot. The interesting fact appeared that the specific protein was present in lower concentration, relative to the total protein, in the two fluids than in the serum. This strongly suggested

the possibility that the specific protein was being taken up by the tumour cells selectively either from these fluids or from the serum which went to form them. It is unlikely that diffusion could explain the relatively low level of the specific protein in these fluids, since it diffuses as rapidly as serum albumin in agar gels. It may be, however, that these fluids contained a large amount of some protein which is either absent from the serum or was undetected by the Ouchterlony method, and this would account for the difference.

Necrosis might be thought to be a factor influencing the increase of the protein, especially as necrotic tumour tissue is known to cause an increase in serum α1-globulin and a decrease in serum albumin and γ-globulin (Dontenwill, Ranz and Mohr, 1959). It is clear, however, that it is not a necessary factor, for high levels of the specific protein occur where there is no necrosis, e.g. in young rats, in pregnant females and in animals undergoing regeneration (Darcy, 1960, 1957). Increases also occurred with tumours which were not necrotic, e.g. some of the August PWA.2 tumours. There simply remains the question of whether necrosis is a contributory factor to the increase in specific protein. Against this is the fact that homografts of normal tissue, which became necrotic, had little or no effect on the protein (Darcy, 1957) and the fact that the August D.177 tumour which becomes considerably necrotic caused only a small increase in titre of the protein and showed no relationship between the amount of necrosis and the level of the protein.

It may be asked whether the level of this protein would be of any use as a diagnostic tool. For tumours in rats the answer is no. Although the level increased dramatically after implantation of the Walker tumour it increased only very slowly with the growth of certain other tumours, and tumours of about 1·5 cm. diameter could be present without an elevation of the level above the normal range. It probably depends on the growth rate of the tumour. As a prognostic tool the level of this protein could conceivably have considerable value, especially in following the effects of treatment and in detecting relapses or metastases. It would be necessary, however, to rule out the interference of other influences, which might be difficult. In any case, the specific protein or an analogous one, has yet to be identified with certainty in human serum.

SUMMARY

1. A quantitative study has been made of an α-globulin in rat serum which had previously been found to increase in association with tissue growth, whether normal or neoplastic.

2. From a normal level of 0·20 units per ml. of serum the protein increased to about 0·72 units at 24 hours after implantation of the Walker tumour and to about 3·0 units at 12 days after.

3. Similar high levels were found with chemically induced tumours although the initial rise was slower.

4. A significant positive correlation was demonstrated between the size of the August PWA.2 tumour and the level of the specific protein in the blood.

5. There was evidence that the protein does not originate at the site of the tumour. There is other evidence compatible with the view that the tumour selectively absorbs the protein from the surrounding fluids.

I should like to express my gratitude to Professor Alexander Haddow for pointing out to me the potentialities of the gel diffusion technique upon which this work is based.

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INHIBITION OF TUMOUR GROWTH BY INTERFERENCE OF HEXOSE MONO-PHOSPHATE PATHWAY. SYNTHESIS AND ANTICANCER PROPERTIES OF THIOPHENE 2:5 DICARBOXYLIC ACID

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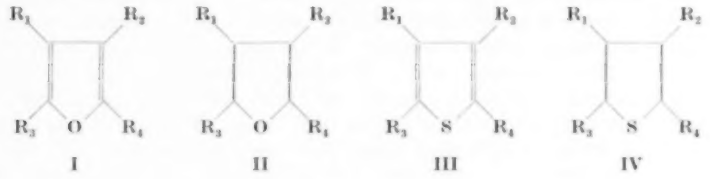
In a previous communication Sahasrabudhe (1958) put forth evidence in support of existence of a chemical competition between nucleic acid and pyridine nucleotide syntheses for the appropriation of a common precursor, adenine. In a rapidly growing malignant tissue, adenine was shown to be appropriated for nucleic acid synthesis and very little was left for incorporation in pyridine nucleotides (Narurkar, Kumta and Sahasrabudhe, 1957). It was further shown that the pyridine nucleotide levels were invariably lowered in presence of rapid nucleic acid synthesis irrespective of whether it (the nucleic acid synthesis) was of neoplastic or non-neoplastic origin (Jedeikin, Thomas and Weinhouse, 1956; Kotnis, Narurkar and Sahasrabudhe, 1959). Pyridine nucleotides have an important role in the hydrogen transport system and thus indirectly participate in the production of energy, via the tricarboxylic acid cycle. In view of this it was suggested that low levels of pyridine nucleotides automatically slow down all the synthetic and proliferative activities by controlling the energy production. Based on these ideas, a biological feed-back mechanism was postulated for the regulation of normal growth processes (Sahasrabudhe, 1958). In tumour tissue however, this feed-back-mechanism seems to break down; the tumour apparently is able to obtain an unlimited supply of energy to maintain its rapid nucleic acid synthesis. A search for an alternate pathway capable of producing energy independent of the proposed feed-back-mechanism revealed that hexose-mono-phosphate pathway (HMP) has the requisite potentiality of not only producing energy (though not yet definitely established) but also yielding ribose-5-phosphate which is the starting material for the biosynthesis of purines. In the light of this the reported preponderance of HMP pathway in tumour tissue acquires greater significance (Kit, 1956; Kit and Graham, 1956; van Vals, Bosch and Emmelot, 1956). Interference of HMP pathway, it was thought, would inhibit the tumour growth by curtailing the supply of energy and ribose-5-phosphate. This has been attempted by preparing anti-metabolites against some suitable intermediates of the HMP pathway.

An ideal chemotherapeutic substance has to have preferential action on tumours only, with no or minimum action on the host tissues. It was this consideration which prompted us to rule out the possibility of useful results with anti-meta-

bolites of glucose-6-phosphate and fructose-6-phosphate (Sahasrabudhe, 1958). These two intermediates are common for both the Embden-Meyerhof (EM) and HMP pathways, and hence the antimetabolites of these would interfere with the HMP and also the EM pathway and thus may prove harmful to the normal host tissue. Antimetabolites against 6-phosphogluconic acid, sedoheptulose and erythrose, however, were free from this objection. In the early stages of HMP pathway the carboxyl group of 6-phosphogluconic acid is liberated as carbon dioxide leaving the rest of the molecular arrangement intact in ribose-5-phosphate. To be effective, the anti-metabolite has to have some functional resemblance to its normal counterpart; it is only then that the anti-metabolite can possibly compete for active sites and block appropriate enzyme systems. The anti-metabolite of 6-phosphogluconic acid therefore should have (i) a free carboxyl group at one end and (ii) a free or phosphorylated hydroxymethyl group or a carboxyl group at the other end. Any deliberate departure then in the structural configuration of central $(CHOH)_4$ grouping of the 6-phosphogluconic acid molecule would result in a substance having anti-metabolite properties. These considerations and also the fact that gluconic acid is readily converted to a 5-membered γ-lactones structure (Fieser and Fieser, 1960) prompted us to suggest that furans, tetrahydrofurans and also thiophenes and tetrahydrothiophene derivatives of type (I), (II), (III) and (IV) might inhibit malignant growth through interference of HMP. It may not be out of place to mention here that 5-hydroxy methyl-2-furfuraldehyde (Heaton and Robinson, 1948) and 5-nitro-2-furfuraldehyde (Friedgood and Green, 1950) have been shown to have potent cancer inhibiting properties.



$R_1 = R_2 = H, OH, OEt$

$R_3 = COOH, COOEt, CH_2OH, CH_2—O—P(OH)(OH)$

$R_4 = COOH, COOEt, CH_2OH, CH_2—O—P(OH)(OH)$

Ribose-5-phosphate is derived in the body not only by the oxidative decarboxylation of 6-phosphogluconic acid; it can also arise through the action of transketolase and transaldolase enzymes. For effective inhibition of ribose-5-phosphate formation therefore, it may be necessary simultaneously to interfere with 6-phosphogluconic acid as well as with erythrose and/or sedoheptulose-7-phosphate. A comprehensive programme of synthesis and testing of anti-cancer properties of compounds of the types mentioned is in progress. The present paper records the synthesis and anticancer properties of thiophene- 2 : 5-dicarboxylic acid (TDA).

EXPERIMENTAL

Synthesis and properties of thiophene-2 : 5-dicarboxylic acid (TDA)

All the four possible thiophene-dicarboxylic acids are known (Hartough, 1952 ; Sice, 1954 ; Kornfeld and Jones, 1954). Among the different methods available for synthesis of thiophene-2 : 5-dicarboxylic acid the two most convenient appear to be the following :—

Method 1.—Thiophene →2 : 5-dichloromethylthiophene →2 : 5-diacetoxymethythiophene →Thiophene-2 : 5-dicarboxylic acid (TDA).

Method 2.—Thiophene →2 : 5-diiodothiophene →2 : 5-dilithium derivative → TDA.

In the present investigation thiophene-2 : 5-dicarboxylic acid was prepared by the second method. The yields by the first method were never more than 21 per cent or so, whereas by the second method the yield was 74 per cent. The various stages in synthesis are as follows.

Preparation of 2 : 5-di-iodothiophene from thiophene

Thiophene (16·8 g.; 0·2 mole) and benzene (20 c.c.) were taken in a glass stoppered flask, and iodine (105 g.; 0·42 mole) and yellow mercuric oxide (75 g.; 0·35 mole) were alternately added in small quantities during 2 hours with constant shaking and occasional cooling to keep the temperature between 30–45°. The yellow mercuric oxide turned crimson red because of its conversion to mercuric iodide. Vigorous shaking is required and if the absorption of iodine is incomplete the mixture has to be shaken for an additional hour. The mixture was filtered and the residue washed with benzene and ether. The ether-benzene filtrate was washed several times with a 3 per cent solution of sodium thiosulfate till it was free from traces of iodine. It was then washed with water and dried over calcium chloride. On removal of the solvent 2, 5-diiodothiophene was obtained as a brownish oil (55 g.). It was distilled at 116–17°/3·5 mm. The small amount of monoidothiophene (2 g.) present was separated as a low boiling fraction. The brownish oil (43 g., 64 per cent yield) gave a red-brown solid on cooling. Crystallization from alcohol gave white plates m.p. 40–41° (Minnis, 1943 ; m.p. 40–41°).

Preparation of phenyllithium (*Evans and Allen*, 1943)

A 250 c.c. three-necked flask was fitted with a mercury-sealed stirrer, a dropping funnel and a reflux condenser. Small pieces of lithium (21 g.; 0·3 gram atom) (prepared by hammering the metal into a thin sheet and cutting it into small thin strips) and 50 c.c. dry ether were placed in it. A slow current of nitrogen was passed before the addition of lithium and ether and the subsequent reaction was carried out under the atmosphere of nitrogen with constant stirring. Dry bromobenzeme (24 g.; 0·15 mole) in ether (20 c.c.) was added slowly during 20 to 30 minutes so that the mixture continued to reflux gently. The mixture formed a gray precipitate of lithium bromide and was stirred for about 1½ hour till it attained room temperature. The contents of the flask were quickly filtered through a cotton-plugged funnel in a 500 c.c. three-necked flask. During this transfer and filtration the mixture was kept under nitrogen.

Preparation of thiophene-2, 5-dicarboxylic acid (TDA) *from 2, 5-diiodothiophene* (*Campaigne and Foye*, 1948)

To the above filtrate (stirred and kept under slight nitrogen pressure) a solution of 2, 5-diiodothiophene (10·5 g.; 0·03 mole) in 50 c.c. ether was slowly added (10 minutes) when a white precipitate separated out. Stirring was continued for 10 minutes and the mixture poured into a beaker containing Dry Ice and allowed to stand for 1–2 hours till all Dry Ice disappeared. The mixture was then acidified with ice cold dilute hydrochloric acid (1 : 1 ; 10 c.c.) and allowed to stand overnight, when most of the ether evaporated off. The white solid (4·3 g.) was filtered and washed with ice-cold water. It was then dissolved in dilute sodium bicarbonate, decolorized with Norit in the cold and filtered. TDA was then precipitated by acidification when a white product (3·5 g.) was obtained. Some more product (0·5 g.) was obtained from the aqueous filtrate, by extraction with ether. The ether solution was again extracted with sodium bicarbonate solution and the purification with Norit and precipitating the product by acidification was carried out as indicated above. The crude acid (4·0 g.; yield 74 per cent) crystallised from water in white needles.

M.P. 322–26° open capillary, shrinking at 318°, effervescence at m.p.

(Found : C 41·8 per cent ; H 2·6, S 18·5 per cent $C_6H_4O_4S$ requires C 41·8 : H 2·3, S 18·5 per cent).

Derivatives—2 : 5-Dicarbethoxythiophene.

M.P. 50–51° (Friedgood and Green, 1950 ; m.p. 51–51°).

(Found C 53·5 ; H 5·4 ; S 14·5 $C_{10}OH_{12}O_4S$ requires C 52·6 ; H 5·2 ; S 14·0 per cent).

Biological testing of TDA

Anticancer activity of TDA was tested on two transplantable tumours (1) rapidly growing Yoshida sarcoma (ascites) in Wistar rats and (2) comparatively slow growing solid fibrosarcoma in Swiss mice. Yoshida sarcoma was obtained through the courtesy of Professor Druckerey (Freiburg). The solid fibrosarcoma used in the present investigation was initially obtained by Waravdekar and Ranadive (1957) from animals treated with 6 : 12 dimethylbenzo (1 : 2-b : 4 : 5-b′) dithionapthene. This has since been maintained in Swiss mice through several serial transplantations.

Influence on Yoshida sarcoma (ascites)

0·5 ml. suspensions of Yoshida ascites sarcoma cells were injected intraperitoneally in 3–4 months old Wistar rats. Thiophene 2 : 5-dicarboxylic acid (TDA) was disolved in 0·5 per cent $NaHCO_3$ (1 mg./0·5 ml.). Since the substance is water soluble it was thought that it would be easily excreted out. Multiple injections per day were therefore tried in an attempt to maintain adequate drug concentration in the system. Secondly for sustained inhibition of tumour growth the concentration of anti-metabolite in the body has to be more than that of its normal counterpart. The dose of anti-metabolite therefore had to be determined depending on the growth rate and the extent of tumour growth. In young animals the growth of the Yoshida sarcoma is fairly rapid and results in the death of all the transplanted animals by about the 4th or 5th day after transplantation. In older animals the growth of the tumour is known to be comparatively slow.

The influence of TDA on slow tumour growth was also investigated by transplanting the tumour in 6–7 months old animals. Three doses were tried : 1 mg. once a day, 1 mg. twice a day (daily dose 2 mg.) and 1 mg. thrice a day (daily dose 3 mg.). Injections of TDA were evenly spaced during the day ; the corresponding control animals received only 0·5 ml. of the solvent (0·5 per cent $NaHCO_3$ solution) intraperitoneally. Survival of treated and control animals was noted. The results are given in Table I.

TABLE I.—*Influence of TDA on Survival of Yoshida Sarcoma (Ascites) Bearing Rats*

Treatment and frequency of injections	Total daily dose (mg.)	Age of rats (months)	Mean survival time (in days)	
			Control*	TDA treated†
(1) 1 mg. TDA ONCE a day	1	3–3½	6·0	6·5
(2) 1 mg. TDA TWICE a day	2	3	4·2	6·0
(3) 1 mg. TDA THRICE a day	3	4½	4·2	6·0
(4) 1 mg. TDA TWICE a day	2	6–7‡	9·5	30·0

* Control group received intraperitoneal injection of solvent (0·5 per cent $NaHCO_3$) only.
† TDA was dissolved in 0·5 per cent $NaHCO_3$ (concentration 2 mg./1 ml.) and administered intraperitoneally.
‡ The growth of tumour in older animals is comparatively slow.

Influence on solid fibrosarcoma

In the case of solid fibrosarcoma the tumour mass was cut into fine pieces with scissors and a homogeneous suspension was prepared in normal saline. 0·5 ml. of the suspension was injected subcutaneously through an 18 gauge needle in each of the Swiss mice. Since the cell count of each inoculum could not be taken, the tumour was allowed to grow in all animals for 7 to 8 days before the treatment was started. On completion of this period animals having comparable tumour sizes (by visual observation) were selected and divided into two groups. The experimental group was injected with TDA dissolved in 0·5 per cent $NaHCO_3$, while the control group received the injections of the solvent (0·5 per cent $NaHCO_3$). In these experiments also the influence of one injection and multiple injections per day was investigated. After 10 to 15 days of treatment the animals were killed and the weights of the tumours determined. The results are given in Tables II and III and in Fig. 1 and 2.

TABLE II.—*Influence of one Injection Per Day of TDA on the Growth of Transplanted Fibrosarcoma in Swiss Mice*

	Daily dose	Frequency of administration	Day of starting treatment after transplantation	Period of treatment (days)	Average weight of tumours and range
Control*	0·5 ml. of 0·5% $NaHCO_3$ only	Once daily	1st	17	2·8 g. (1·56–4·65 g.)
TDA-treated†	1 mg. TDA in 0·5 ml. of 0·5% $NaHCO_3$ solution	,, ,,	1st	17	2·1 m.g. (1·59–2·57 g.)

* Control group was injected with the solvent (0·5 per cent $NaHCO_3$) only.
† TDA was dissolved in 0·5 per cent $NaHCO_3$ (concentration 2 mg. TDA/ml.).

TABLE III.—*Influence of Multiple Injections per day of TDA on the Growth of Transplanted Fibrosarcoma in Swiss Mice*

	Daily dose	Frequency of administration	Time of starting treatment after transplantation (days)	Period of treatment (days)	Average weight of tumours and range
Control*	0·5 ml. of 0·5% $NaHCO_3$ only	Twice daily	10	15	8·2 g. (6·36–9·18 g.)
TDA-treated†	2 mg. TDA dissolved in 0·5% $NaHCO_3$ solution	1 mg. twice daily	10	15	3·16 g. (1·79–3·90 g.)

Tumour was allowed to grow for 10 days. Then the animals having uniform tumour sizes were divided into control and experimental groups.

* The control group was injected with corresponding volume of the solvent, i.e. 0·5 per cent $NaHCO_3$ solution.

† TDA was dissolved in 0·5 per cent $NaHCO_3$. Every time 1 mg. dissolved in 0·5 ml. of 0·5 per cent $NaHCO_3$ was injected.

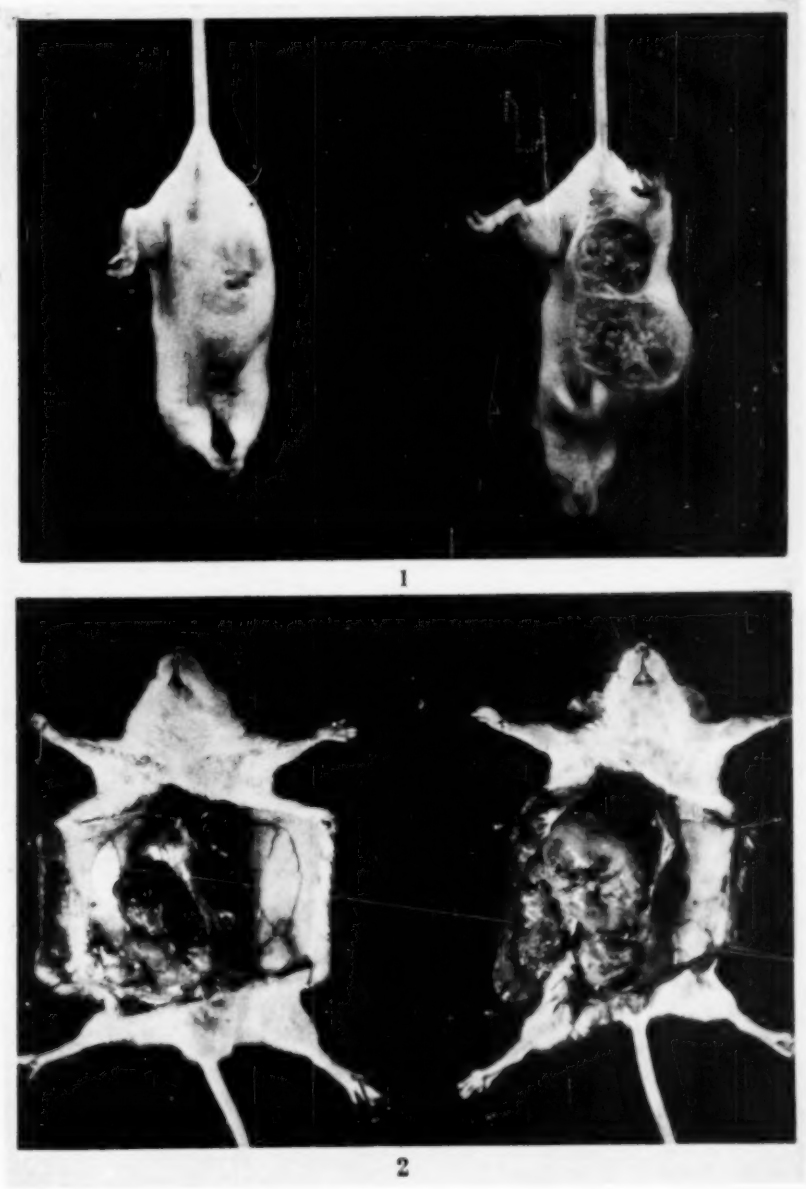
In vitro *studies on interference of HMP*

In addition to the *in vivo* screening of antitumour activity it was necessary to ascertain whether TDA really interfered with the HMP pathway. This has been investigated in *in vitro* studies by incubating the tumour tissue with glucose-1-^{14}C and glucose-6-^{14}C, with and without the presence of TDA. Six to eight weeks old Swiss mice weighing between 20 and 25 g. were used. Solid fibrosarcoma was transplanted subcutaneously. Tumours were dissected out three weeks after transplantation and homogenised in Potter Elvehjem glass homogeniser in 6 volumes of ice cold medium containing 0·133 M phosphate buffer (pH 7·4). Aliquots representing 80 mg. of tumour tissue were added to chilled Warburg vessels. The incubation medium consisted of the following substances expressed in their final concentrations (Wenner and Weinhouse, 1956) :—Potassium fumarate 7×10^{-5}M ; cytochrome C 4×10^{-6}M ; DPN 2×10^{-3}M ; phosphate buffer (pH 7·4) $6{\cdot}0 \times 10^{-3}$M ; $MgSO_4$ 3×10^{-3}M ; KCl $1{\cdot}4 \times 10^{-1}$M ; glucose 0·020 M and glucose-1-^{14}C or glucose-6-^{14}C (as the case may be) equivalent to $6{\cdot}42 \times 10^4$ cpm. 1 mg. of thiophene 2, 5-dicarboxylic acid dissolved in 0·5 c.c. of 0·5 per cent $NaHCO_3$ was added to the medium. A rolled filter paper soaked in 0·2 ml. 10 N NaOH was placed in the central well of Warburg flasks to absorb the CO_2 released. 0·3 ml. 50 per cent trichloroacetic acid was placed in the side arm. The flasks were attached to manometers and the assembly shaken for 4 hours. At the end of this period, trichloroacetic acid was tipped in the reaction vessels to stop the reaction and to liberate CO_2 from the incubation medium. The flasks were shaken for an additional period of 60 minutes to allow complete absorption of CO_2 by the alkali. The manometers were then detached and the

EXPLANATION OF PLATE.

FIG. 1.—Inhibition of growth of transplantable fibrosarcoma with TDA treatment. Dose 2 mg./day. Treatment started 10 days after transplantation and continued for 15 days. Left hand—treated with TDA ; right hand-control.

FIG. 2.—The same animal as in Fig. 1. With the tumour exposed. Note the inhibition of growth in the TDA treated animal. Left hand—treated with TDA ; right hand-control.



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filter papers and the rinsings of the central well with 0·5 per cent $NaHCO_3$ were carefully transferred to test tubes and made up to a known volume. Aliquots of this were taken for plating and counting with a thin window G.M. counter (1·5–2 mg./cm²). The results are given in Table IV.

TABLE IV.—*Influence of Thiophene 2, 5-Dicarboxylic acid on the Liberation of Radioactive $^{14}CO_2$ from Glucose-1-^{14}C Glucose-6-^{14}C Substrates by Tumour Tissue*

Substrate	Additions	Radioactive $^{14}CO_2$ given out
Glucose-1-^{14}C	Nil	127
Glucose-1-^{14}C	Thiophene-2,5-dicarboxylic acid	71
Glucose-6-^{14}C	Nil	45
Glucose-6-^{14}C	Thiophene-2,5-dicarboxylic acid	26

RESULTS AND DISCUSSION

It will be seen from Table I that there was not much difference in the survival of the control and treated animals when only single injection of TDA (1 mg.) was given every day. When the frequency of injections and daily dose was increased, the TDA treated animals survived longer. When the dose and frequency of daily injections were increased and at the same time the growth of the tumour was slowed down (as in old animals) the results were striking. In this group the treated animals survived up to 30 days whereas the control animals died by the 12th day after transplantation.

Similar results were obtained with the solid fibrosarcoma in Swiss mice. When the daily dose was 1 mg. once only the weights of the tumours in the control untreated animals were not markedly different from those of the treated animals. But when the frequency and the daily dose was increased, significant inhibition of tumour growth was evident in the TDA treated animals. Thus whereas the mean weight of the tumour in the control animals was 8·0 g. that of the treated groups was 3·0 g. This is clearly seen in the photographs of the treated and untreated animals (Fig. 1 and 2).

In the *in vitro* studies with glucose-1-^{14}C and glucose-6-^{14}C it was seen that liberation of radioactive $^{14}CO_2$ from glucose-1-^{14}C was diminished in presence of TDA suggesting that the HMP was probably inhibited by the presence of TDA. It will be noticed that in the presence of TDA the liberation of radioactive $^{14}CO_2$ from glucose-6-^{14}C substrate was also diminished. This is due to the fact that in tumour tissue a portion of trioses is obtained via the HMP pathway. If this is so then the diminution in the evolution of radioactive CO_2 when glucose-6-^{14}C is used is to be expected. *In vitro* studies thus seem to justify the premise that TDA or similar substances would inhibit the HMP pathway and thus bring about inhibition of tumour growth.

SUMMARY

Inhibition of tumour growth with anti-metabolites of hexose-mono-phosphate (HMP) intermediates has been attempted. Thiophene 2 : 5-dicarboxylic acid (TDA) has been synthesized and its tumour inhibiting properties have been investigated on Yoshida (ascites) sarcoma in rats and on transplantable solid

fibrosarcoma in Swiss mice. The survival of Yoshida sarcoma bearing rats was increased with TDA treatment, whereas with the solid fibrosarcoma a significant inhibition of tumour growth was evident. *In vitro* studies carried out with glucose-1-^{14}C and glucose-6-^{14}C suggest that TDA interferes with the HMP pathway.

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AN EXPERIMENTAL METHOD FOR COMPARING TREATMENTS OF INTACT MALIGNANT TUMOURS IN ANIMALS AND ITS APPLICATION TO THE USE OF OXYGEN IN RADIOTHERAPY

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WHEN the results of radiobiological experiments (Gray, Conger, Ebert, Hornsey and Scott, 1953) first led to the use of oxygen with radiotherapy for human cancer (Churchill-Davidson, Sanger and Thomlinson, 1955) the need for a satisfactory experimental system for comparing differing schemes of treatment became apparent. A system using malignant tumours in the rat for comparing the results of many forms of cancer therapy has been developed. This will be described together with the results of the first of a series of experiments using radiotherapy and different conditions of oxygenation.

The Principles of an Experimental System

It cannot be over emphasised that in using a biological experimental system, the variables between one test and another should be the relatively simple and measurable physical and chemical factors, whilst the effect on the complex and usually immeasurable biological factors should be made to match. In the context of this investigation the question is being asked " to what extent does the concentration of oxygen reaching a neoplasm modify the dose of radiation required to produce a particular result? "

Answers to this question can never be obtained from human radiotherapy since one patient varies so much from another and no two neoplasms are exactly alike. Moreover there are narrow ethical limitations to the variation of treatment and in general only one result may be sought. If however, the experimental testing of therapy in animals is to influence clinical practice, the pathological principles underlying the experimental system and the human situation must be understood and be seen to be identical ; the more nearly the experimental conditions simulate those of the treatment of human cancer the better.

For these reasons it has seemed essential to use an animal neoplasm which infiltrated and gave metastases because these are the two outstanding characteristics of the disease of cancer. The use of a pure in-bred strain of animal maintained by " brother-sister " mating ensures that each experimental animal is as like another as a mammal can be. A transplantable tumour arising or induced within the strain, whilst open to some criticism, makes it reasonable to regard different instances of the growth as in essence the same neoplasm. Finally, a randomising process must be introduced to determine the treatment to be given in each instance and the results must be susceptible to statistical analysis.

The Technique of Transplantation used for Producing Tumours for Experiment

The difficulty of using a malignant neoplasm is that metastases may form before the treatment is given and lead to the death of the animal before the effect of treatment on the primary tumour can be assessed. After many failures to obtain results for this reason a somewhat elaborate technique which appears to overcome the difficulty has been evolved.

Rats have been used for these experiments because of their convenient size. They are of "John's strain", derived from a single pair of Wistar siblings in 1939. The tumour used so far is a fibrosarcoma induced in the strain by subcutaneous injection of benzopyrene in 1945 and known as RIB5.

The tumour has been maintained by subcutaneous grafts in the flank. From such a graft, healthy tumour tissue is minced with scissors and mixed with a solution of sodium alginate in the approximate proportions of two parts of tumour to one of alginate. This mixture is then dropped from a fine hypodermic needle into a 1 per cent solution of calcium chloride. A gel of calcium alginate is formed so making a capsule to the drop, the thickness of which increases with time. As the technique is now used, 20–30 seconds is sufficient to produce a tumour "pellet" which can be handled in a pipette and transferred after this time to a physiological saline solution.

Meanwhile a "sausage skin" has been prepared by taking the small intestine of a freshly killed three week old rat, inverting a length of it and wiping off the mucosa with a gauze swab. Tumour "pellets" are now placed within the lumen of the inverted intestine which is tied on either side of each pellet and cut into sections to produce almost spherical tumour "sausages" each about 3 mm. in diameter.

A skin incision just large enough to admit a pipette containing the tumour sausage is made in the abdomen, and then a burrow in the subcutaneous tissue layer immediately deep to the panniculus carnosus extending as far as the position in the flank where the tumour is to grow. A tumour sausage is then put into the blind-end of the burrow with a pipette and the skin incision closed.

Most of the tumours transplanted in this way grow to form rounded masses, in the clinical sense unattached to skin or muscle, reaching a diameter of 10 mm. in about fifteen days (Fig. 1).

EXPLANATION OF PLATES.

FIG. 1.—A rat with a solitary, localised, smooth and rounded subcutaneous tumour in the flank. This is a random example 9 mm. in diameter.

FIG. 3.—Photograph of pressure chamber with upper hemisphere removed. The gas inter-tube and needle valve are in the foreground. Beneath the chamber is the box containing the heating coil and behind it, the cooling columns. Two lead colimater rings are on the box and one is in place above the couch which supports the rat. On the right is the box containing the electrically-operated valves controlling gas flow and in front of this lies the "U"-shaped clamp for making the tumours anoxic.

FIG. 14.—Photograph of part of the cut surface of a fixed tumour RIB5 about 40 mm. in diameter. The edge of the tumour is on the left. Near this is a zone of homogeneous intact tumour. On the right all the tissue is necrotic. In the intermediate zone are many prominent, dilated and congested blood vessels.

FIG. 15. Photomicrograph of tissue of tumour RIB5 from the intermediate zone shown in Fig. 16. What was homogeneous tissue has broken up into cylindrical systems of about 100 μ radius, with a dilated blood vessel at the centre. These blood vessels have walls of endothelial cells and basement membranes only. Surrounding them is a zone of intact tumour tissue. Outside this is a zone of necrotic tissue in which cells are clearly recognisable and in which the necrosis is relatively recent. Beyond this all the tissue is necrotic and details are lost. This necrosis is older.

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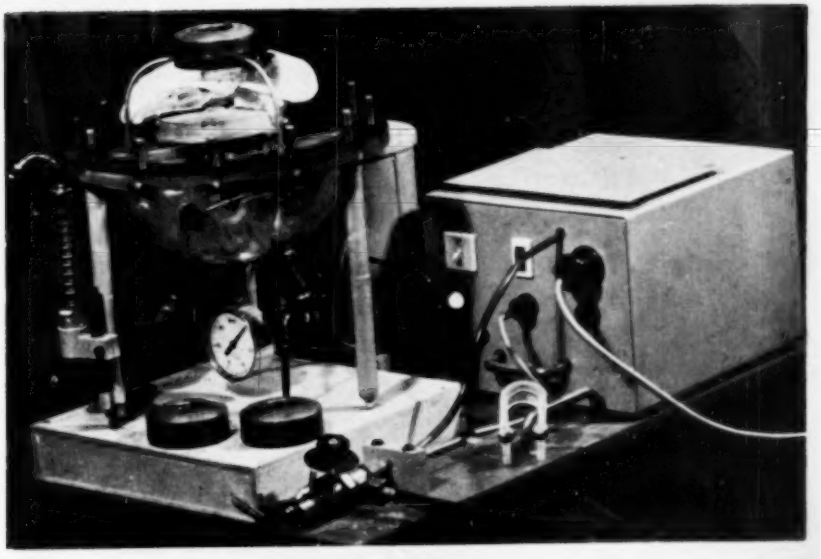

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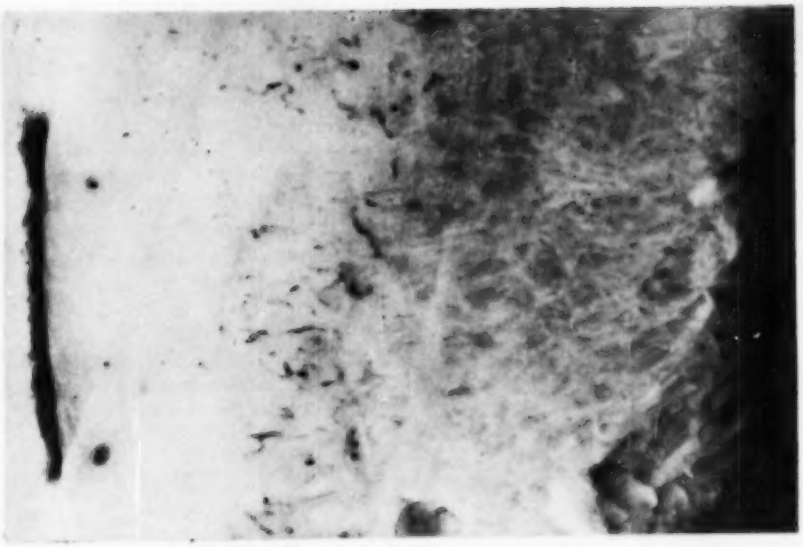

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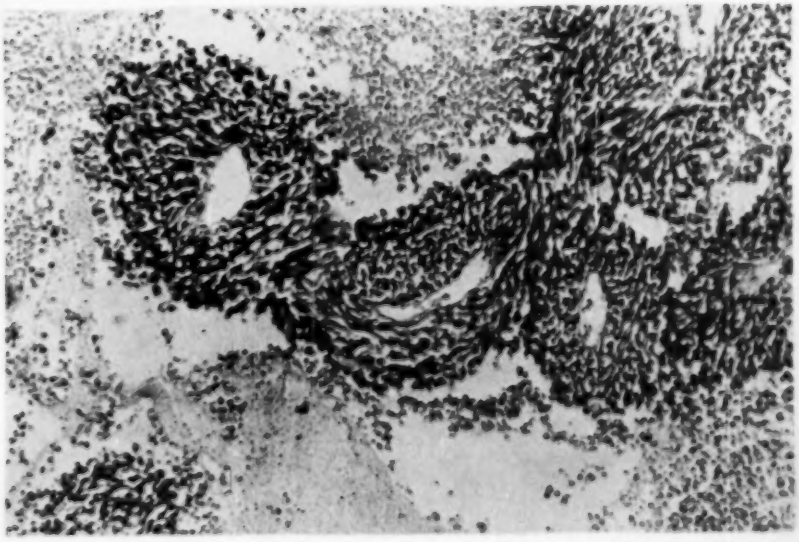

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The Technique for Irradiation Tumours in Differing Pressures of Oxygen

All irradiations were made with a Marconi Industrial Model X-ray set, with an electrically operated shutter, running at 250 kv. and 10 or 15 m.a. to give a dose rate of 400 rads per minute using filters of 0·5 mm. copper and 1·0 mm. aluminium. The mid-point of the tumour was approximately 21 cm. from the target of the X-ray tube. The dose given at each irradiation was controlled by using a monitor placed in front of the shutter. This consisted of an ionisation chamber through which the X-rays passed and the current of which was integrated. This current was correlated with the dose received at the position of the mid-point of the tumour as measured with a condenser-ionisation chamber. All the animals were treated within a spherical pressure chamber 9 inches in diameter

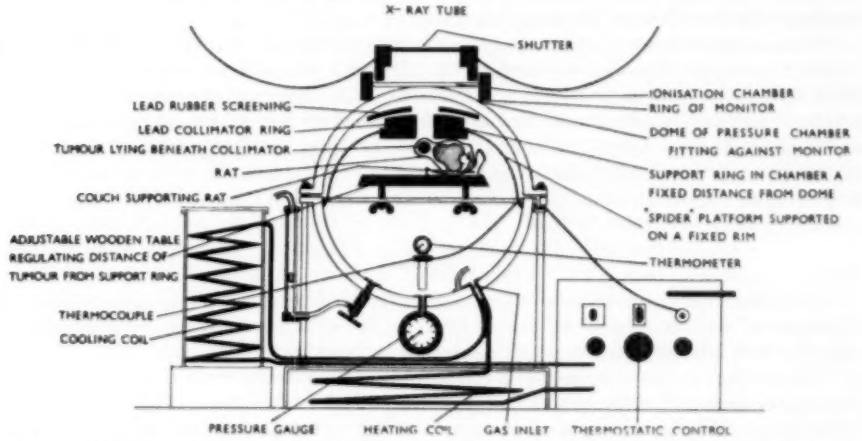

FIG. 2.—Diagram of the pressure chamber for treating rats at high pressures of oxygen, showing the mechanisms for controlling gas flow and temperature, the method of supporting and protecting the rat and the relation of the chamber to the monitor and X-ray tube.

made from $\frac{1}{2}$ inch Perspex sheet moulded into two hemispheres with flanges, which were bolted together through a rubber gasket (Fig. 2 and 3).

The temperature within the chamber was kept at 30° C. $\pm$ 2° C. by passing the incoming gases either through a coiled pipe heated electrically or through a water cooled spiral. These alternative channels were controlled by electrically operated valves governed by a thermostat within the chamber. Mixing of gases in the chamber was ensured by a baffle just within the entrance port. When oxygen was passed into the air-filled chamber at 10 l./min. less than 0·5 per cent nitrogen, measured by a mass spectrometer, was present after two minutes. The gas pressure was regulated by a British Oxygen Company constant pressure delivery valve on the gas cylinders, and a needle valve near the chamber. This pressure was indicated by a pressure gauge on the chamber. The gas flow was regulated by a needle valve on the exit from the chamber with a gas flow-meter beyond this.

The anaesthetised rat was placed on its side on a couch in the upper part of the chamber so that the tumour lay free of any external support or traction.

This couch was attached to a wooden platform carried on a Perspex "spider" resting in turn in the lower hemisphere of the chamber. Also carried by this "spider", immediately above the rat, was a horizontal metal ring about the vertical axis of the chamber. In this ring could be placed one of several rings of lead, one inch in thickness, used to define the X-ray beam. This lead ring was therefore in a fixed position in relation to the lower hemisphere of the chamber and hence the upper hemisphere when that was in place. The upper hemisphere was brought into relation with the monitor on the outlet of the X-ray tube. The distance that the tumour lay from the X-ray tube was adjusted by a vertical movement of the couch and wooden platform in relation to the lead defining ring. A single measurement from the mid-point of the tumour to the under surface of the defining ring was all that needed to be made in calculating the monitor reading which indicated the selected dose.

It was possible by palpation to define the position of the deep surface of the tumour and the adjacent abdominal muscle and this was marked on the skin. With the animal lying on its side, the centre of the tumour was placed in the axis of the chamber in such a way that a verticle beam of X-rays was tangential to the abdominal musculature. A suitably sized defining ring was chosen to ensure irradiation of the whole tumour and protection of the intestine. The rest of the animal was covered by a sheet of lead rubber.

Procedure

The rats were immobilised by anaesthesia produced with an intra-peritoneal injection of sodium amylobarbitone, 12·5 mg. for a 200 g. rat, and proportionately slightly less or slightly more with lighter or heavier animals.

When irradiation was to be given with the tumour anoxic a clamp made of two horse-shoe shaped pieces of Perspex was placed on the skin around the tumour, between it and the abdominal muscle and tightened to occlude the circulation. The animal was placed on the couch in the pressure chamber, which was then closed and air passed through it at a rate of about 1 litre per minute. The clamp was left in position for six minutes before radiation began and removed immediately after its completion.

For irradiation to be given with the animal breathing air sodium amylobarbitone was given in the same way and the rat placed in the pressure chamber with no interference to the tumour circulation. The chamber was closed and air passed through it. Irradiation was given immediately. On some occasions, after completion of irradiation, oxygen was given at pressure in the manner used for irradiation in oxygen.

When irradiation was given with the animal breathing oxygen at high pressure it was anaesthetised and placed in the chamber in the usual way. Oxygen was passed through the chamber at 10 l./min. to flush out the nitrogen. The pressure within the chamber was then raised at a rate of 10 lb./min. The earliest rise in pressure sometimes caused movement of the animal. This could be stopped or minimised by raising the pressure more slowly. The extent of any movement could be seen from the marks on the skin, and if need be the chamber was opened and the process started again. The pressure of oxygen was raised to 45 lb. per square inch (4 atmospheres absolute) and maintained for fifteen minutes before irradiation.

Immediately before and immediately after irradiation in each condition the position of the tumour was inspected and the respiration rate measured. During treatment the animal was observed visually and the thermometer, pressure gauge and gas flow meter could be seen.

After the completion of irradiation in oxygen the pressure was lowered slowly. After using pressures of 30 lb. per square inch five minutes appeared to be a long enough time, but after 45 lb. per square inch pressure thirty to forty minutes were required, if damage to lungs or nervous system were to be avoided.

The Design of the Experiment

This experiment was designed to test the hypotheses that in an intact malignant tumour there are cells which are not fully oxygenated, that these, after aerobic irradiation, are capable of regenerating the tumour in its environment and that they can be influenced by the breathing of higher pressures of oxygen.

The effect of irradiating tumours was therefore tested at two doses, 2000 rads and 4000 rads, and under each of three conditions, (i) the tumour being made anoxic by stopping its circulation, (ii) the tumour " aerated " with the rat breathing air, and (iii) the tumour " oxygenated " by the rat breathing oxygen at 45 lb. per square inch pressure. Results were obtained by making daily measurements of the diameter of the tumour in each of the three dimensions with graduated calipers and taking the arithmetic mean of these three measurements.

In addition to these irradiation " treatments " six types of control " treatment " have been carried out. In the first, only daily measurements were made. In the second, no irradiation was given but the animals were given the anaesthetic. In the third, the anaesthetic was given and the circulation to the tumours was occluded for twenty minutes. In the fourth the anaesthetic was given and the animal placed in oxygen at 45 lb. per square inch pressure for twenty minutes. The fifth type of control treatment was surgical excision of the tumour performed to test the frequency of metastasis formation before the time of treatment. The sixth control treatment consisted of giving the rats oxygen at 45 lb. per square inch pressure for 20 minutes after irradiation in air or after irradiation of the tumour in the anoxic condition.

Measurement of the growing tumour could be made from diameters of about 5 mm. upward. All treatments were given when the tumours had attained a mean diameter of between 8 mm. and 10 mm. Each form of treatment was given a number and was allotted to the tumours from a table of random numbers as they reached this size. The pre-requisite conditions for any treatment to be given were that the tumour was solitary, smooth, rounded and mobile in the subcutaneous tissue and clinically not attached to skin or muscle.

RESULTS

The results of this experiment are based on the treatment of 82 tumours. Of these two animals were lost by death from haemorrhage into the lungs immediately after decompression in oxygen, when this had been done too quickly. A third animal having a doubtful additional minute nodule outside the field of radiation was discarded when this grew within a few days of treatment. A fourth was discarded from the surgical series because of local recurrence within one week.

TABLE I

Type of treatment	Number of animals	Mean diameter of tumours (mm.)
Untreated control	8	9·4
Control given amylobarbitone only	3	8·8
Control given amylobarbitone and clamping of circulation	3	8·6
Control given amylobarbitone and oxygen at 45 lb. per sq. in. pressure	3	8·6
Surgical excision	10	9·1
2000 rads given to anoxic tumours	6	9·5
2000 rads with rat breathing air at atmospheric pressure	7	9·1
2000 rads with rat breathing oxygen at 45 lb./sq. in.	9	9·0
4000 rads with tumour anoxic	6	8·5
4000 rads with rat breathing air at atmospheric pressure	9	9·0
4000 rads with rat breathing oxygen at 45 lb./sq. in.	14	8·8

Of the remaining seventy-eight tumours, the distribution amongst the different forms of treatment and the mean diameter of each group at the time of treatment is shown in Table I.

It should be noted that whilst the numbers in some of the control groups are still small these can be added to in subsequent experiments. The larger number of those treated with 4000 rads given in oxygen is due to an additional six being treated in succession at the end of the experiment.

Result of surgery

In all 11 tumours were excised. In one animal there was local recurrence at the site of the operation. On post-mortem examination no metastases were found. One of the remaining ten animals died on the 50th day after excision. In this animal there was no sign of neoplasm at the site of operation or in the axillary, inguinal or iliac lymph nodes. However, there were massive metastases in the lungs, in the upper mediastinum and around the lower part of the pericardium and upper surface of the diaphragm. The remaining nine animals are well and without sign of desease after more than 120 days from the time of excision.

Results of control and radiation treatments

In this first experiment it has seemed worth presenting the curves relating the mean diameter of each tumour to the day before or after treatment (R day) to show the extent of the variation. These curves are shown in Fig. 4 to 11.

In the untreated control group (Fig. 4) all tumours grew at a nearly uniform rate. Of the six animals shown, two died probably from haemorrhage into the tumour, before a diameter of 50 mm. was reached. In the second control group (Fig. 5) the growth rate of all tumours, whether treated with anaesthetic only, anaesthetic and clamp or anaesthetic and oxygen, was similar and fell within the range of the untreated controls. After 2000 rads given with the tumour made anoxic (Fig. 6) the growth rates were similar and slightly but appreciably delayed as compared with the control. After 2000 rads given whilst the animal was breathing air with the tumour circulation unimpaired (Fig. 7) there was considerable delay in growth and more variation from tumour to tumour. This variation was more prominent after 2000 rads given with the rat breathing oxygen at 45 lb/square inch pressure (Fig. 8). One tumour of this group became impalp-

able after 29 days and the animal is well after 120 days. At the other extreme the growth rate of one tumour was similar to that of the anoxic group.

After 4000 rads given to the anoxic tumour (Fig. 9) there was once more less variation in response as compared with the air and oxygen groups and an appreciable slowing of growth as compared with 2000 rads given to the anoxic tumour. 4000 rads given with the rat breathing air (Fig. 10) resulted in some variation of

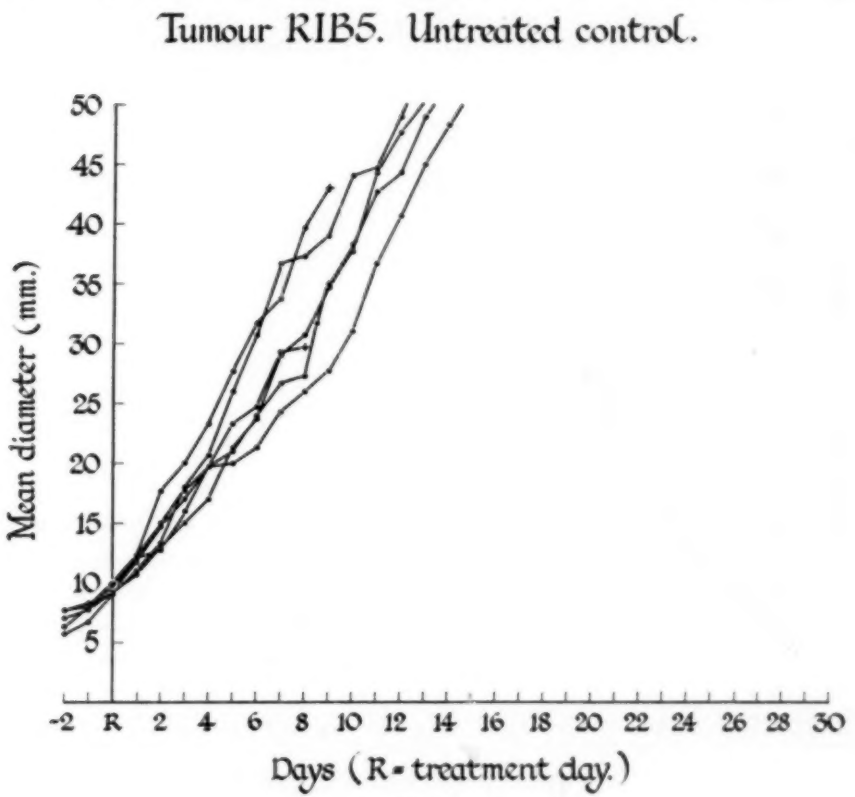

FIG. 4

response but there was a common trend of all but two of the nine tumours included. In the last group treated with 4000 rads whilst breathing oxygen (Fig. 11) the different response appeared to have resulted in two populations. One of these composed of four tumours had behaved like the anoxic group, whilst the other ten had followed a common trend, usually resulting in continued growth, but with two tumours disappearing for over 120 days up to the time of writing.

Possible reasons for these different responses will be discussed. Meanwhile the results of these different treatments may be compared from the means either of all the tumours in each group (Fig. 12) or, as seems more reasonable, those in

each group which follow a common trend (Fig. 13). It will be noted how the variation within each group increases from the anoxic group through the air group to the oxygen group and is more marked with the higher dose than the lower.

The mean survival time of all animals in each group is shown in Table II.

Not all tumours grew after irradiation to reach the large size of 50 mm. in diameter. Three tumours were apparently cured. Many other animals died of

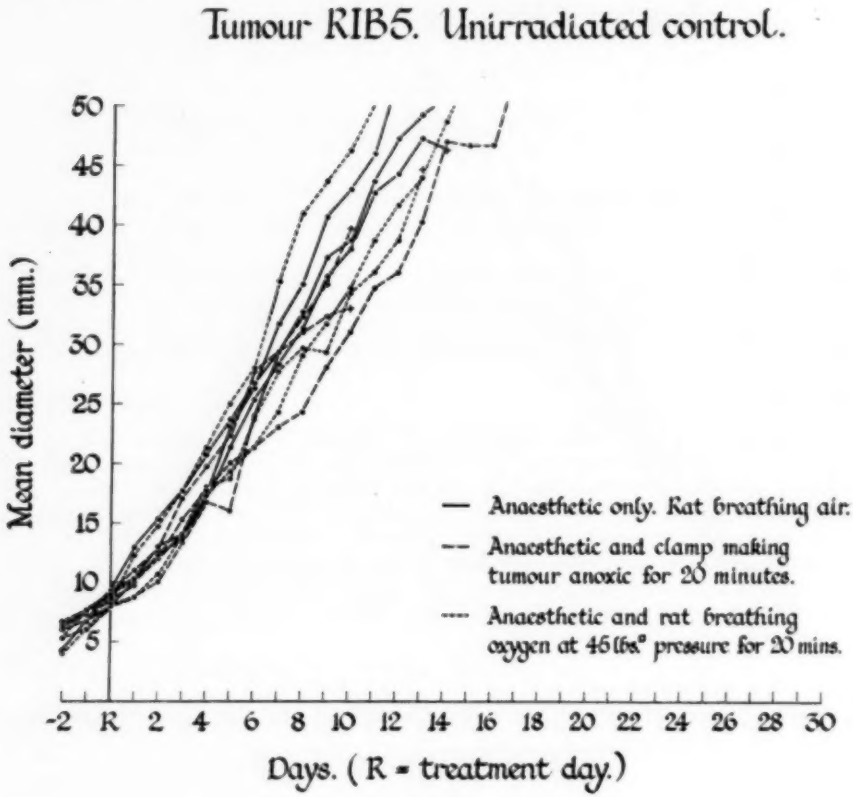

FIG. 5

TABLE II

Treatment	Mean survival time in days
All controls	$17 \pm 1 \cdot 1$
2000 rads, tumour anoxic	$21 \pm 1 \cdot 6$
2000 rads, rat breathing air	$28 \pm 1 \cdot 3$
2000 rads, rat breathing oxygen	$39 \pm 8 \cdot 2$
4000 rads, tumour anoxic	$27 \pm 1 \cdot 7$
4000 rads, rat breathing air	$37 \pm 0 \cdot 8$
4000 rads, rat breathing oxygen	$45 \pm 7 \cdot 1$

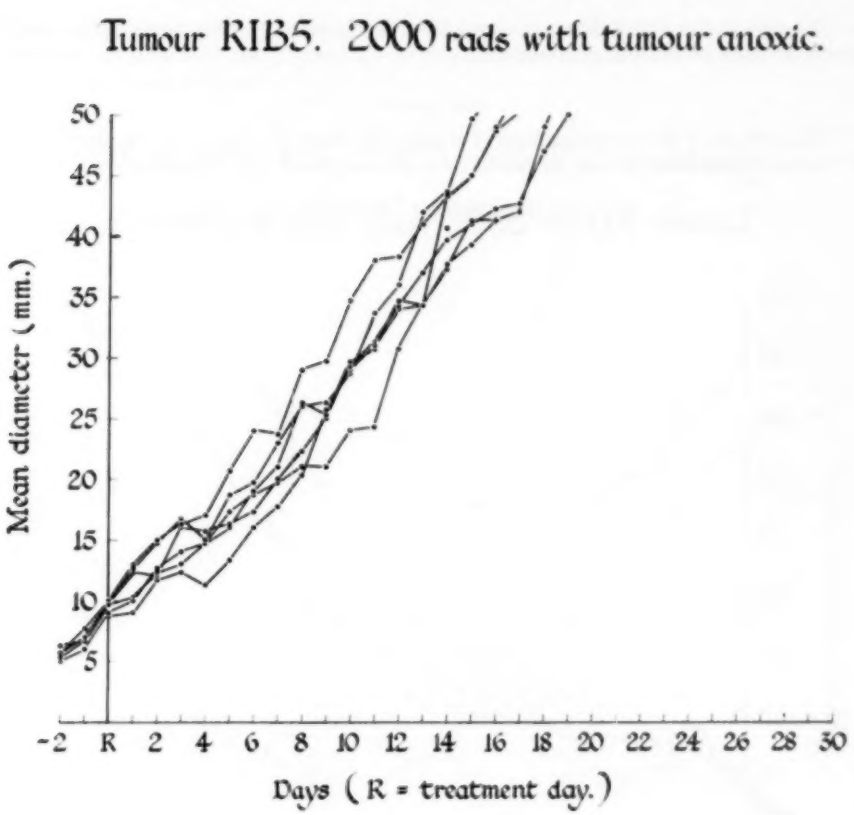

FIG. 6

metastases before the tumours had grown large. However, in each group several tumours have grown to 50 mm. in diameter, and the average times they have taken to do this are listed in Table III.

TABLE III

Treatment	Time after treatment day for growth to 50 mm. diameter in days
Controls	14 (29 after implantation)
2000 rads, tumour anoxic	18
2000 rads, rat breathing air	26
2000 rads, rat breathing oxygen	36
4000 rads, tumour anoxic	26
4000 rads, rat breathing air	38
4000 rads, rat breathing oxygen	46

No significant result has appeared from analysis of postmortem findings and these will not be presented in detail.

DISCUSSION

The object of the investigations of which this paper is a report is the elucidation of certain problems to do with the use of oxygen in combination with radio-

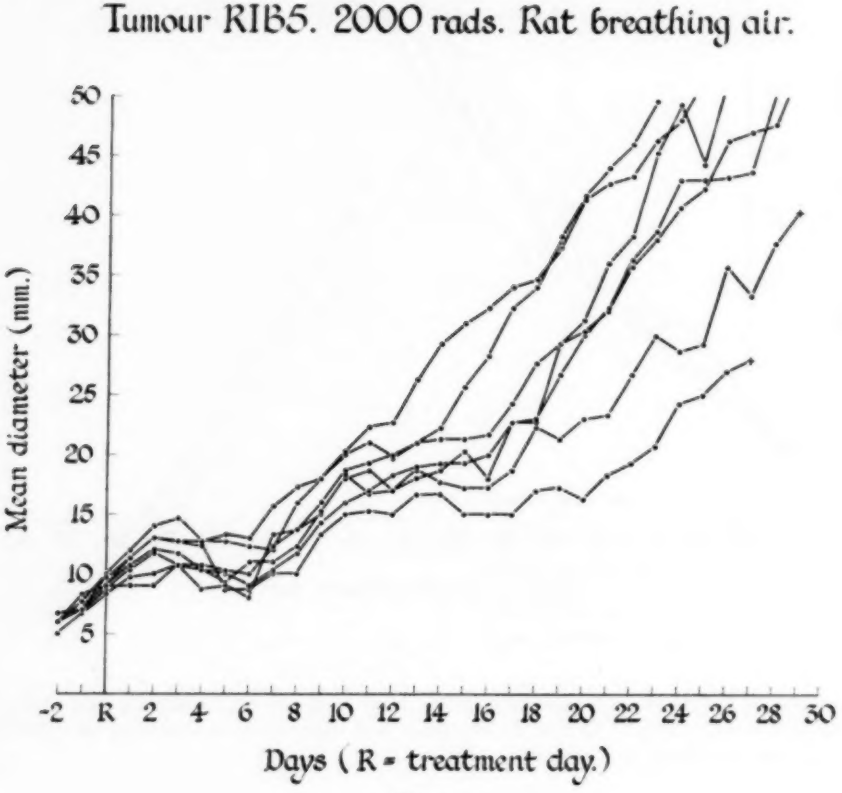

FIG. 7

therapy in the treatment of human cancer. The first answer to be sought is a clear demonstration of the beneficial effect of oxygen with radiation in the treatment of a tumour irradiated and followed *in situ* in the host animal with no further interference than the making of daily measurements. Other practical questions such as the optimal pressure of oxygen which should be breathed, the minimal time for which oxygen should be given before treatment, the whole complex issue of the fractionation of the total dose, and the dose itself in relation to tumour size and type are awaiting answers. Clearly these will only come in the

light of understanding of the radiobiology of neoplastic cells, the pathology of tumour growth and the physiology of oxygen transport in the animal body and within the tumour itself.

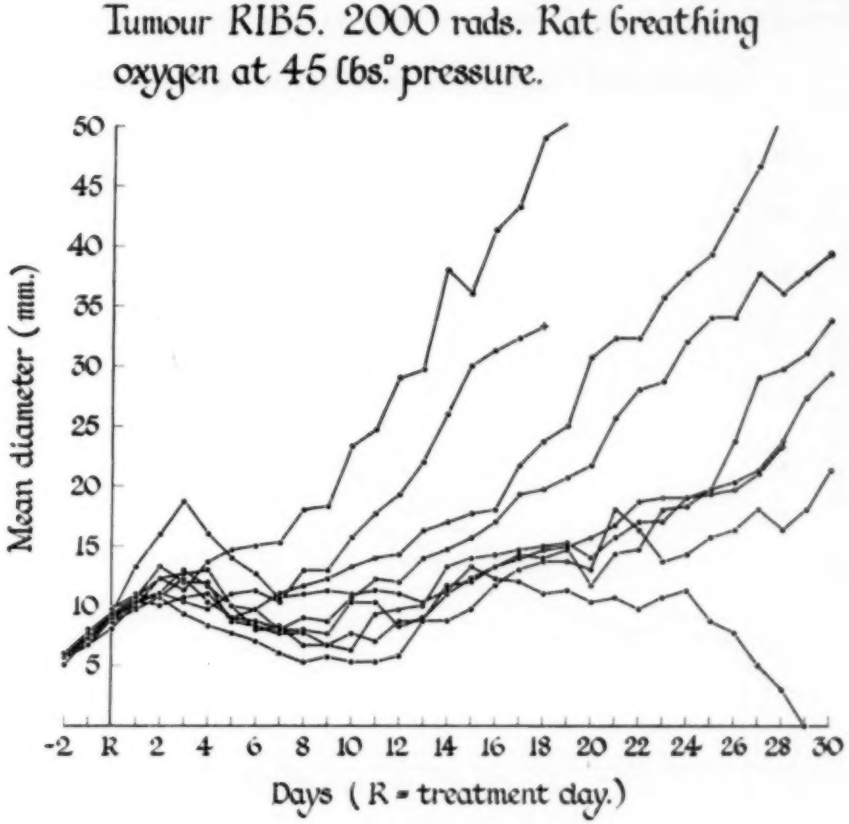

FIG. 8

The pathological basis of the experiments

Radiobiological aspects of the effect of oxygen on systems of cells, neoplastic and otherwise have been extensively studied and recently reviewed (Alper, 1960; Gray, 1958; Scott, 1958). It seems probable that all nucleated cells will show a similar relationship between oxygen availability at the time of irradiation and sensitivity to radiation injury as judged by damage to the reproductive integrity of the cells. Similarity between the cells of the experimental sarcoma RIB5 used in the experiments reported here and, for example, the Ehrlich mouse ascites

tumour (Deschner and Gray, 1959) has been assumed, but must be confirmed in due course.

Direct evidence of the presence of cells at low concentrations of oxygen in intact tumours at the time of irradiation is scanty and uncertain. The probable existence of oxygen gradients in certain types of carcinoma such as would be likely to cause low levels of oxygen availability to some cells has been suggested

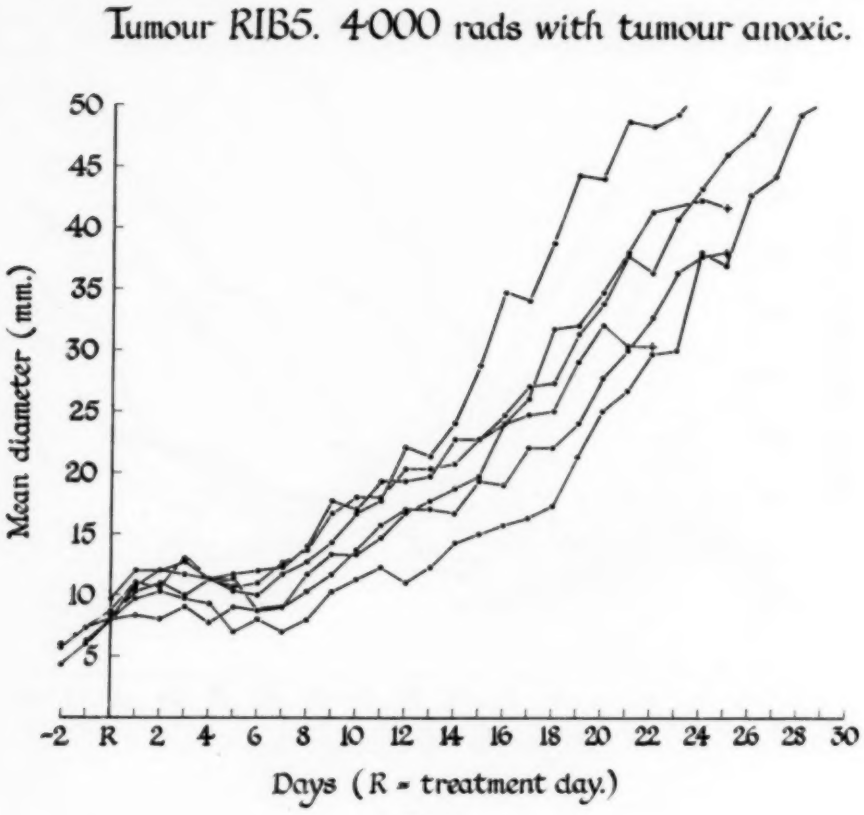

FIG. 9

(Thomlinson and Gray, 1955). The type of diffusion pattern which they reasoned must exist was considered in relation to the circulation and necrosis in other types of tumour by Churchill-Davidson, Sanger and Thomlinson (1957).

The importance of this relationship lies in the frequency with which a common pattern of vascular disturbance and necrosis is found in most types of malignant growth including human cancer. For example, when tumour RIB5 has grown to a large size, say 30 mm. or more in diameter, the whole centre has undergone coagulative necrosis and at the periphery is an irregular zone of intact tumour

tissue ranging from 5 mm. to 10 mm. in thickness. In the outer part of this zone there is no necrosis. A little towards the centre, small areas of necrosis appear at points at the greatest distance from blood vessels. In the region bordering on the necrotic centre the intact cells appear to break up into cylindrical systems surrounding dilated and congested blood vessels, the walls of which are of capillary structure (Fig. 14 and 15). At the periphery of these systems are zones of necrotic

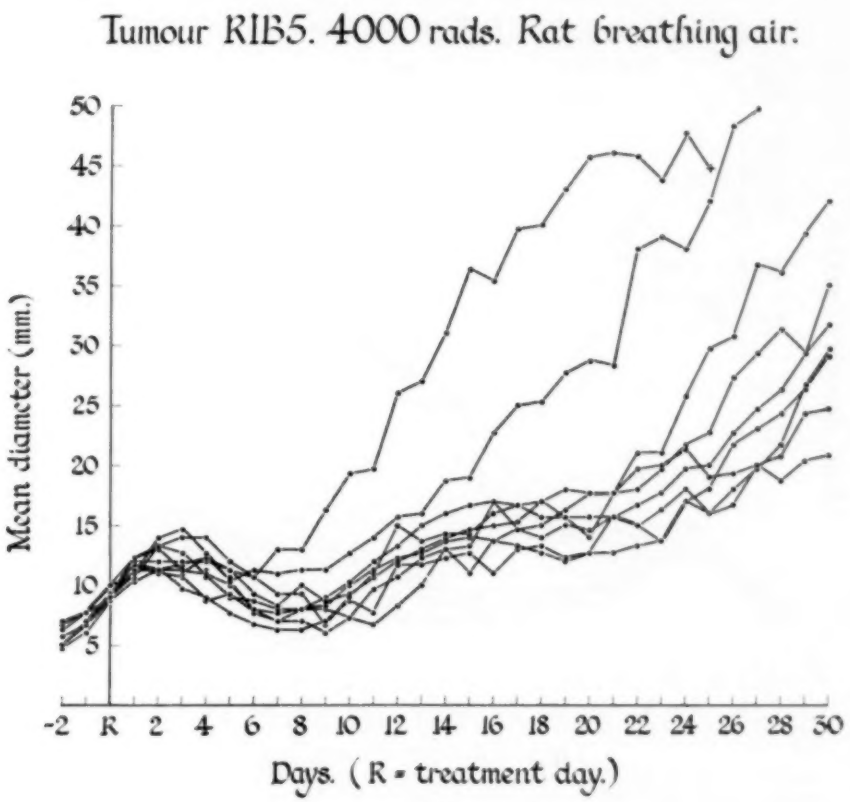

FIG. 10

cells where cell membranes are yet intact and cell structure is visible. At greater distances from the central capillary the whole tissue is necrotic and no cellular detail is visible. In a map of such a region (Fig. 16) the uniformity of width of intact cells around each capillary and the regularity of the zone of necrotic but recognisable cells suggests a dynamic system in which there is a gradual shrinking of the amount of tissue nourished by each capillary. This in turn suggests a gradual progressive failure of the circulation within these capillaries. It is hard to believe that there are not gradients of oxygen tension falling with distance from

such capillaries. It seems reasonable to suppose that living cells in these tumours which are in low concentrations of oxygen, if they exist, are to be found at the greatest distances from such capillaries, that is to say, on the borders of necrotic areas.

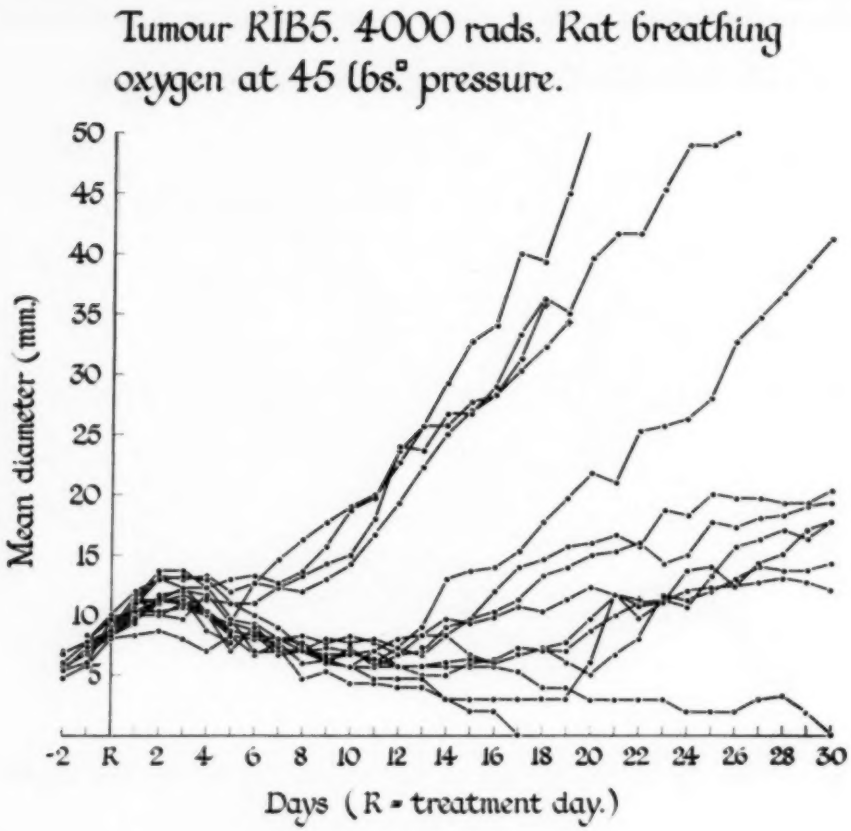

FIG. 11

If this picture of the presence and position of hypoxic cells in a tumour is right, it seems that in the natural course of events their " days are numbered " and it may be supposed that they are of no concern to the radiotherapist since they cannot become " more dead than dead " (Bevan). However, it is a hypothesis that after irradiation in aerobic conditions these cells may survive because of the protective effect of hypoxia and regenerate the tumour in the better nutritional conditions which follow the death and absorption ot their more radiosensitive neighbours.

John's hypothesis (see Churchill-Davidson, Sanger and Thomlinson, 1957) suggests the massive necrosis in tumours is due to venous infarction following slowly progressive venous obstruction consequent upon the expansion of the tumour mass in a limited space. If this were correct it would imply that more oxygen

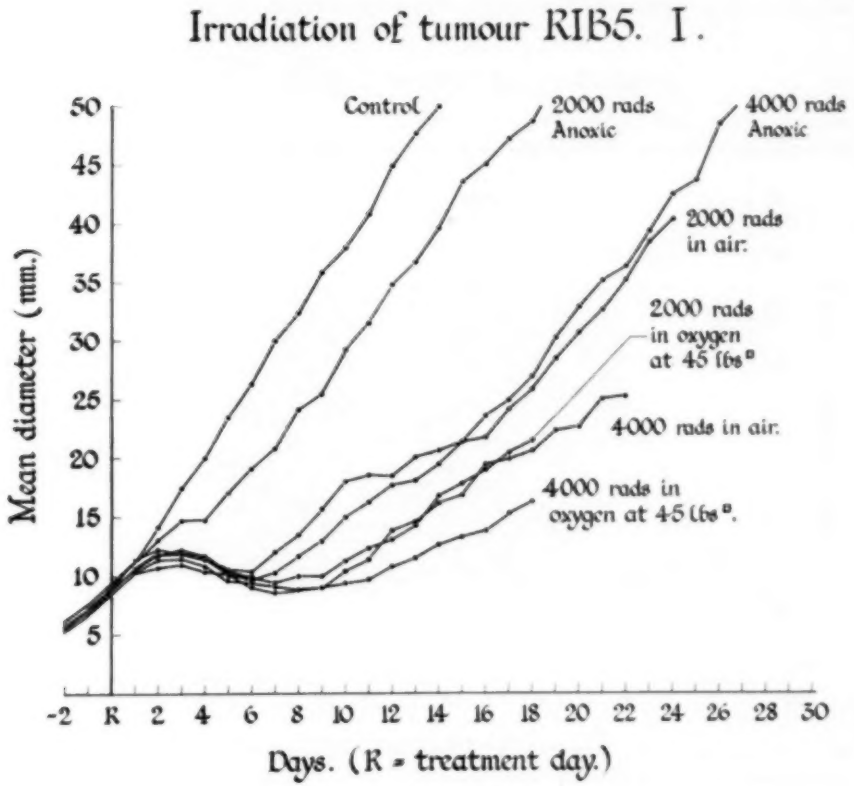

FIG. 12.—The arithmetic means of the growth curves of all tumours in each group. Increased oxygen tension, from anoxic conditions through breathing air to breathing oxygen, results in increased damage and delayed growth of the tumour with each dose. Some curves stop earlier than the 30th day because of the death of a few animals with large tumours. Note the matching of the curves after 4000 rads given to the anoxic tumour and 2000 rads in air and those of 4000 rads in air and 2000 rads in oxygen.

can only reach regions in which it is deficient if each volume of blood were to carry more, since further vasodilation would not increase the blood-flow. This was the basis upon which the breathing of oxygen at high-pressures was introduced at St. Thomas's Hospital.

In developing an experimental system to study the use of oxygen it seemed essential to use a neoplasm which grew to form a pattern of necrosis and vascular

change similar to that found in human neoplasms and also which extended by infiltration and metastasis formation. The latter is important because it is at least possible that the profound inflammatory changes following irradiation may influence the spread of the disease by this means.

A transplantable tumour is the only type of neoplasm which permits these factors to be studied quantitatively. The objections to the use of transplantable

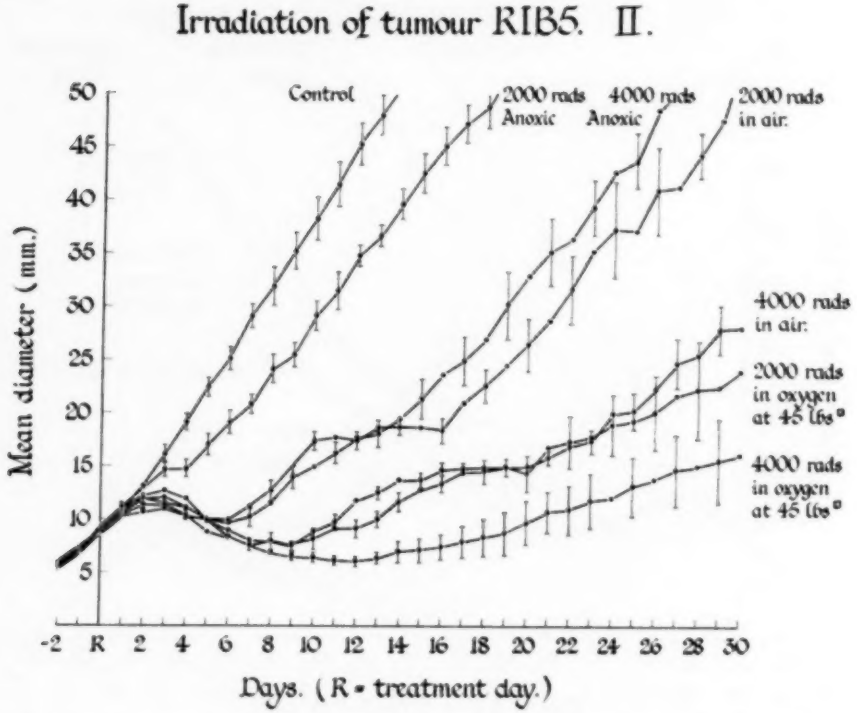

FIG. 13. The arithmetic means of the growth curves of those tumours in each group following a common trend. The standard errors of the means are shown. Note the match between the curves after 4000 rads given to the anoxic tumour and 2000 rads given in air and those of 4000 rads given in air and 2000 rads in oxygen.

tumours lie in the realms of tumour immunology. It may well be that no such tumour is isogenic with its host (Prehn and Main, 1957). However, the influence of immunity can be minimised by using in-bred strains of animal and tumours arising spontaneously or by induction within the strain. The degree of incompatibility can be tested by using the cell dilution techniques of Hewitt (1958) and the four tests suggested by Scott (1960) are being carried out. The effect of immunological incompatibility between tumour and host is to produce cures after irradiation when cells have survived the radiation injury in such numbers as

would have regenerated the tumour if no immune reaction existed. The relations between radiation damage and immunological response are very complex and have recently been discussed (Scott, 1960). These inter-relations make the exact comparison of the groups in these experiments rather difficult but the possible effects of immunity are diminished when comparison is made between dose and oxygen concentration which produce equal damage to the tumour.

Transplantation technique

The development of a technique which would admit this type of comparison has proved surprisingly difficult. At first, small masses of apparently healthy solid tumour were implanted subcutaneously, but these resulted in irregular

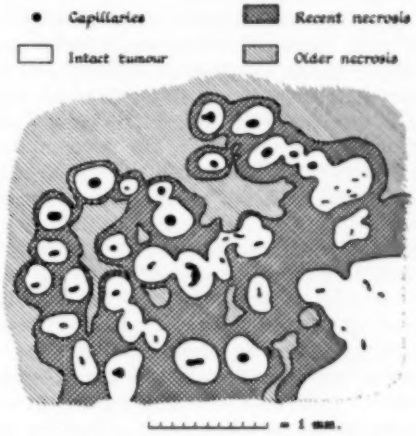

FIG. 16.—A diagrammatic map of tissue in the intermediate zone as shown in Fig. 14 and 15. The uniform width of the zone of "recent necrosis" suggests a dynamic system with gradually failing circulation in the "capillaries".

shaped tumours which frequently grew into skin or muscle. Centrifuged free cell suspensions were then injected into subcutaneous tissue through a fine hypodermic needle, but these tumour cells followed the needle track and the incidence of early lymph node metastases was high.

At this stage a mixture of a centrifuged cell suspension and sodium alginate was first used in proportions of two parts to one. This mixture was dropped into a 1 per cent solution of calcium chloride and left in it for varing lengths of time. It had been determined that the tumour cells alone would grow after ten minutes immersion in this solution. It was found that after 20 seconds immersion the calcium alginate formed at the periphery of a drop of the mixture produced a capsule such that the tumour pellet could be handled by pipette and be implanted in the subcutaneous tissue. However, whilst almost all these "pellets" grew to form tumours many of these followed the needle track and infiltrated the skin. The impression was formed that the "pellets" were too fragile and ruptured after implantation. At the other extreme "pellets" left in calcium chloride for five

minutes were almost solid and failed to grow at all. Eventually a time of 1¼ minutes was selected as giving a reasonable yield of tumours (about 75 per cent of those implanted).

Irradiation experiments carried out with those tumours resulted in a disappointing loss of usable material within a few days following treatment either from the death of animals due to metastases or the rupture of tumours through the skin.

An extensive series of surgical excisions of the primary tumours at varying sizes from 3 mm. to 12 mm. in diameter resulted in 50 per cent cures irrespective of size. This indicated that metastasis formation, or spread of the tumour beyond the field of excision and therefore of irradiation, had occurred in the other 50 per cent of cases as a result of the transplantation technique. Experiments starting from this situation seemed fruitless.

During the course of these experiments it was realised that those tumours which, at the size of 10 mm. diameter, felt smooth and rounded, and which were in the clinical sense mobile in the subcutaneous tissue and unattached to skin or muscle, went on to grow to a large size without obvious metastases before a late stage in the disease. This seemed to be the condition at the 10 mm. size in which the tumours were suitable for experiment. The problem appeared to be to hold the transplanted cells together in a mass until the trauma produced by the transplantation process had healed, or at any rate until a barrier had formed around the transplant.

Recollecting, from the days of keeping pigs during the war, the way of making sausage skins from small intestine, it was decided to try to make viable tumour " sausages ". In the first place the viability of tumour cells within a capsule of intestinal wall was tested by scraping off the mucosa of the adult rat jejunum and filling the lumen with minced tumour. This was then tied into suitable lengths and implanted. The tumours all grew and histological examination showed the presence, not only of viable cells, but of newly formed blood vessels both within and without the remnants of the intestinal wall. In spite of washing and searing it is quite possible that many viable tumour cells were left beyond the " ties " at the end of each sausage, and therefore not surprising that the resultant tumours were of irregular shape. The next step, however, yielded satisfactory results. This was to implant fragile alginate tumour pellets into the lumen of the inverted intestine of a young rat. The pellets held the tumour cells together until the ties had been made. These spherical " sausages " have given a good yield of tumours meeting the conditions required for experiment. A few have infiltrated muscle but this was probably due to implantation in too deep a subcutaneous layer. The results of the small series of surgical excisions performed so far indicate that at the 8 mm. to 10 mm. size the chances of the tumour being still localised are high. It seems likely that this technique can be used for the implantation of other types of tumour and will enable *in vivo* comparisons to be made of other types of cancer therapy as well as radiation.

Irraduation technique

Little comment need be made on the radiation technique except to emphasise the necessity of avoiding any manipulations which may impair the circulation in the tumour, bearing in mind the fragile structure and the low intravascular pressures of the veins. Any question of interference with these invalidates the

results of experiments involving oxygenation. Variations of skin circulation with environmental temperature should also be borne in mind.

The need to reduce the pressure in the chamber very slowly after irradiation in oxygen became apparent when two animals developed spastic paraplegia of the upper limbs. This was probably caused by some embolic phenomenon in the spinal cord and although there has been recovery, it is not complete.

The apparatus used in these experiments is being modified to demonstrate radiographically the presence of the whole tumour in the radiation field immediately before and after the dose is given.

Results

The most disturbing feature in the results presented from this first experiment is the scatter of the growth curves following irradiation in air and more still in oxygen. This is more obvious with the higher dose and most pronounced in the separation into two populations seen after 4000 rads. given in oxygen. Although this variability is explicable in terms of small differences in the number of cells surviving radiation the different response may come to reflect the difference between cure and failure in treatment. Five possible explanations may be advanced.

First, that the tumours growing more rapidly after irradiation in fact contained a rather larger number of viable cells at the time of irradiation. Provided that all the cells are well oxygenated, where two out of fourteen tumours have been completely killed by 4000 rads, all would be expected to be killed by 5000 rads. The effect of the small possible difference in numbers at the time of irradiation would disappear in this case, and the result of experiment with a higher dose will decide this explanation.

Second, that the whole tumour was not irradiated. Whilst this seems unlikely, steps are being taken to demonstrate radiographically that the whole of each tumour is in the radiation field at the beginning and end of treatment.

Third, that parts of the tumour remained hypoxic in spite of oxygen administration. This seems the most likely explanation and quantitative predictions of the effect of the presence of a few anoxic cells are consistent with these results. (Hewitt, 1959). The use of different pressures of oxygen may resolve this. It is possible that damage to the lungs from breathing oxygen (Bean, 1945) might have impaired the oxygenation of the arterial blood, either because of pulmonary oedema or intrapulmonary shunting of blood. However, local hypoxia in the tumour seems a more likely explanation than general arterial hypoxia.

Finally, a radioresistant strain of cells can be postulated, but this seems unlikely (Conger, 1956 ; Nice, 1957).

The requirement of the experiment to produce matching results with differing doses of irradiation and conditions of oxygenation has been approximately achieved. Whether comparison is made between all tumours in each group (Fig. 12) or those in each group following a common growth pattern (Fig. 13) two pairs of growth curves are nearly the same. The effect of 4000 rads given to the anoxic tumour is roughly equivalent to the effect of 2000 rads given to the tumour with the rat breathing air. The effect of 4000 rads given to the tumour with the rat breathing air and 2000 rads to the tumour with the rat breathing oxygen at 45 lb/square inch pressure are nearly equal. Also the difference between one pair and the other is statistically significant.

Since the whole growth pattern of the tumours has been disturbed by any of these forms of radiation, it may be doubted that the matchings of one growth curve with another at any particular point is valid. Very good matches could be obtained between all the curves within the first five post-irradiation days! It therefore seems worth while to consider and later to investigate the factors which govern the shape of any of these curves.

Clearly the curves are composite, representing on the one hand the rate of removal of dead tumour tissue, and on the other the multiplication of surviving tumour cells. The curve of the rate of removal of dead tissue will also be composite, because dead cells lying amongst capillaries with an active circulation are eliminated very much more rapidly than a necrotic mass which has to be reorganized. Both these processes are likely to be affected by the effects of radiation on the capillaries.

A number of possibilities affect the shape of the curve representing multiplication of the tumour cells surviving radiation.

The linear relationship between the radius of the tumour and time in the control curve suggests that cell death is taking place in the larger tumours at about the same rate as cell production. In these tumours the whole central region is necrotic and is surrounded by a viable rim. If this rim maintains a constant thickness, which approximately it does, its volume increases five-fold as the radius of the tumour doubles. Since the time taken to double the radius is about four and a half days and the number of intact tumour cells is proportional to the volume of the rim, the generation time of these cells is slightly less than one day.

The curve of tumour size after irradiation with 4000 rads in oxygen shows a doubling of the radius in twelve days, beginning on the fourteenth post-irradiation day. This might indicate a generation time of two and a half days—an unlikely delay of metabolic processes in cells surviving the first two weeks. However, the growth rate may be reduced by nutritional deficiency due to the effects of radiation on capillary blood-vessels. Another possibility is that many cells have suffered less than lethal genetic damage and that in a series of cell divisions damaged material is gradually eliminated in non-viable daughter cells. In this way the total number of viable cells and therefore the tumour mass might remain almost constant for a long period.

In the animals which survive long enough for the tumour to reach the large size of 50 mm. diameter the growth curves gradually steepen and come almost or quite equal to the slope of the control group. The times taken to reach this size are shown in Table III. The longest time was 46 days after the dose of 4000 rads in oxygen. This may be compared with the control group which reached this size 14 days after the treatment day and 29 days after implantation. The difference between 29 days and 46 days might be explicable in terms of the number of cells surviving the implantation process in the first case and the number surviving irradiation in the second. If this is so, the shape of curves representing the cell multiplication processes could be identical and the times taken by each group to reach the large size would be proportional to the number of cells surviving. It is interesting that on this basis there is also a close match between the group receiving 4000 rads in anoxic conditions and 2000 rads in air, and the group receiving 4000 rads in air and 2000 rads in oxygen. It will be of interest to investigate the various possible factors influencing the shape of the curves.

These results confirm those of earlier workers with " solid " tumours (Holcroft,

Lorenz and Matthews, 1952; Scott, 1953; Dittrich and Stuhlmann, 1954; Grüssner, 1957; du Sault, Eyler and Dobben, 1959). Whilst no great mathematical precision should be attached to the ratio of 4 : 1 shown in the effect on the tumour with the rat breathing oxygen compared with the anoxic tumour the results do support three conclusions:

1. In the tumour RIB5 there are cells which are protected by anoxia from radiation damage whilst the animal is breathing air.

2. After irradiation "in air" these cells are capable of multiplying to regenerate the tumours, and

3. The radiation injury to these cells is enhanced by giving the rat oxygen to breathe at 45 lb. pressure.

The physiological and pathological mechanisms bringing about these effects are equally likely to apply in human tumours as in rat tumours where the same patterns of growth and circulatory disturbance are found. It is therefore likely that the use of oxygen in the radiotherapy of human cancer will diminish the number of cancer cells surviving a given dose of radiation and increase the proportion of patients cured.

SUMMARY

A technique has been developed for growing transplantable malignant tumours in the subcutaneous tissue of the rat in such a way that they remain localised until they have grown to a suitable size—10 mm. diameter—for testing the effects of different treatments. The course of the tumours was followed *in situ* by daily measurement. Comparisons have been made of the effects of single doses of 2000 rads and 4000 rads of 250 kv. X-rays under three different conditions of oxygenation of the tumour; with the tumour made anoxic by clamping the circulation, with the tumour "aerated" with its circulation intact and the rat breathing air at atmospheric pressure and with the tumour "oxygenated" with the animal breathing oxygen at 4 atmosphere's pressure. The effect of 2000 rads given in air approximately equals that of 4000 rads to the anoxic tumour and the effect of 2000 rads in oxygen approximately equals that of 4000 rads in air. These results indicate that when the rat breathes air there are cells in the tumour protected from radiation injury by hypoxia; that after radiation in air such cells can regenerate the tumour and their radiosensitivity can be enhanced by the breathing of oxygen at high pressures. The pathological basis of these conclusions suggests that they apply equally to many forms of human cancer.

I should like to thank Sir Robert Davis for the gift of the pressure chamber used in these experiments, Dr. J. B. West of the Post Graduate Medical School for measurements with the mass spectrometer, Mr. D. Moore for measurements of radiation dose, my technician Mr. J. Whitaker, and other members of this Unit, almost all of whom have helped to make the work possible.

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A STUDY OF DOSE AND EFFECT IN INITIATION OF SKIN TUMOURS BY A CARCINOGENIC HYDROCARBON

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In the study described in this paper, we have attempted to learn how the incidence of tumours elicited in mouse skin by initiating and promoting stimuli (reviewed by Salaman, 1958) is related, quantitatively, to the amount of carcinogenic hydrocarbon absorbed by the skin, rather than to the amount applied.

Methods have been devised (McCarter, 1956) to confine the applied hydrocarbon to a measured area of the skin of an immobilized mouse and to limit the amount absorbed by limiting the time that excess hydrocarbon remains on the skin. Some animals are killed so that the amount of the hydrocarbon absorbed by the skin can be determined. Others are allowed to live and are treated topically with croton oil to produce tumours.

Using these techniques, it was possible to show (McCarter, Szerb and Thompson, 1956) that the number of tumours produced in the skin of a mouse varied directly with the area of skin covered by the hydrocarbon, with the time allowed for absorption and with the logarithm of the concentration of the solution applied (9,10-dimethyl-1,2-benzanthracene in liquid paraffin). Subsequently (McCarter, 1958), an attempt was made to relate the tumour incidence to the amount of hydrocarbon (3,4-benzopyrene) that penetrated unit area of skin in a given time following the application of a solution of the substance in acetone. The present paper describes a similar, but more extensive investigation using 9,10-dimethyl-1,2-benzanthracene, hereafter called DMBA.

METHODS

Application of hydrocarbon to the skin

The animals used were female mice of the CFW strain (Carworth Farms Inc., New City, New York) 8 to 10 weeks of age. They were housed in groups of 10 in acrylic plastic boxes with stainless steel tops. The bedding was sawdust. Water and Purina Fox Chow Cubes were freely available.

Each animal was immobilized by the intraperitoneal injection of a solution of Meprobamate (Miltown, kindly supplied by Dr. F. M. Berger, Wallace Laboratories, New Brunswick, N.J.) using 0·40 g. per kg. body weight. This was prepared as an 0·8 per cent solution in 5 per cent gum acacia. A second dose of 0·2 g. per kg. body weight was given 4 hours after the first to those animals which were to be immobilized for 7 hours.

Hair was removed from the back using electric clippers. Only those mice were used that were in the resting phase of the hair cycle, as indicated by the lack of a "fuzzy" appearance after clipping and, in those animals kept for tumour

production, lack of hair growth during the next few days. This technique might have allowed a few animals early in the growth stage to have been included among those used for the chemical analyses, but we wished to avoid any complications that might arise by prior treatment of the skin, by plucking, for example.

A solution of DMBA in redistilled acetone was applied to a circular area of $2{\cdot}3 \pm 0{\cdot}3$ sq.cm. (Standard Deviation) on the mid-portion of the back using the applicator described by McCarter (1956). The mouse was then placed until required in a dark incubator maintained at 25° C. Then, the excess of hydrocarbon remaining on the skin was carefully removed by washing with diethyl ether (McCarter, 1956). The animal was returned to its cage to await further treatment with croton oil, or was killed so that chemical analysis of the dosed area of skin could be made.

Production of tumours

Beginning three weeks after treatment of the animal with DMBA, each mouse was painted twice weekly with 2·5 per cent croton oil (Bush and Co., Canada) in liquid paraffin (paraffin oil, Fisher Scientific Co., Montreal, Canada ; Viscosity 125/135, N.F.) using an artist's No. 6 camel hair brush. The solution was spread over an area greater than that treated with DMBA. The appearance of the back of the mouse was recorded in a sketch once each week (usually checked by another observer) so that the time of appearance and subsequent history of each tumour could be noted. Any tumour larger than 2 mm. in diameter (approximately) and observed on 3 consecutive weeks was recorded.

Chemical analysis

The animal was killed by a blow on the head. The position of the circle treated with DMBA was made visible by brief illumination with an ultraviolet light having the major part of its emission at 366 mμ. The treated area of skin was excised together with a narrow untreated margin.

A suitable number of such treated circles (5 for contact-times of 1 hour or longer ; 10 for times less than 1 hour) were pooled and extracted in a Soxhlet apparatus with 95 per cent ethanol (80 ml.) for 24 hours. Under these conditions, extraction of the hydrocarbon from the skin was complete. The ethanol extract was heated under a reflux condenser with 2 ml. of 50 per cent potassium hydroxide for 6 hours to saponify lipids. After cooling, the mixture was diluted with water and extracted repeatedly with petroleum ether (b.p. 30 to 60° C.) in a specially-constructed glass U-tube (so that contamination with grease et cetera could be avoided). The petroleum ether extracts were combined and evaporated to dryness under reduced pressure.

It was necessary to separate the hydrocarbon from substances in the extract that interfered with the measurement of the ultraviolet absorption spectrum. The residue derived from the evaporation of the petroleum ether was dissolved in a mixture of petroleum ether and benzene in the proportions 80 : 20 (v/v) and the solution was transferred to a chromatographic column of Florisil, 9·7 g., 60 to 100 mesh in a tube 2·2 cm. in diameter. (Florisil, Floridin Co., Tallahassee, Florida). The solvent mixture was passed through the column and collected in 10 ml. portions. Those that contained DMBA were located using ultraviolet absorption spectroscopy, pooled and evaporated to dryness under reduced pressure. The

residue was dissolved in 95 per cent ethanol and transferred quantitatively to a suitable volumetric flask. The ultraviolet absorption spectrum of the solution was measured relative to that of a similarly prepared control solution derived from a quantity of skin equal to that used in the analysis and obtained from mice dosed on the skin with acetone and washed with diethyl ether. Silica cells having a light path of 1 cm. were used in the Beckman DK-2 Ratio Recording Spectrophotometer. The absorption spectrum from 260 to 340 mμ. provided qualitative identification of DMBA and the concentration was calculated from the molar extinction coefficient at 297 mμ. (E_{molar} at 297 mμ. = 4·90).

Using the analytical procedure described above, amounts of DMBA varying from 0·6 to 6·4 μg. added to mouse skin were recovered as noted in Table I.

TABLE I.—*Analytical Recovery of Amount of DMBA Added to Mouse Skin*

Added (μg.)	Recovered (μg.)
0·6	0·6
2·2	2·2
3·2	3·4
6·4	6·3
6·4	6·4
6·4	6·0

RESULTS

In experiments to determine the amounts of DMBA in the skin after washing the excess from the surface, different results were obtained by different experimenters. The data of Table II illustrate these results. It is apparent that,

TABLE II.—*Amounts of DMBA in Skin of Mouse at Various Times After Application of Solution 0·25 per cent in Acetone. Means of 4 Analyses, 10 Circles per Analysis for Times Less Than 1 Hour; 5 for Times Greater Than 1 Hour. Standard Errors. Area, 2·3 sq.cm.*

Time (hours)	Investigation B (μg.)	Investigation H (μg.)
0·25	1·09 ± 0·05	0·35 ± 0·03
0·5	1·05 ± 0·13	0·35 ± 0·08
1	1·40 ± 0·04	1·47 ± 0·13
3	1·89 ± 0·12	2·73 ± 0·25
5	2·09 ± 0·06	3·73 ± 0·16
7	..	4·18 ± 0·06

although agreement between the two sets of results was not achieved, each experimenter could reproduce his own results with accuracy (as indicated by the standard errors). For reasons which are considered in the Discussion, experiments were undertaken to learn if variation in the amounts of hydrocarbon applied to the skin could influence its absorption.

A volume of 0·15 ml. of an acetone solution of DMBA of concentrations 0·005 to 1·0 per cent was applied to an area of 2·3 sq.cm. of the skin of the back of an immobilized mouse (5 in each group) using the procedures described above. Three hours later, the excess of hydrocarbon on the skin was removed and the

treated areas were excised, pooled and analyzed for their content of DMBA. The results of this experiment are recorded in Table III. It is apparent that the

TABLE III.—*Influence of Variation in Amount of DMBA Applied to Skin on Amount that Penetrates in 3 Hours. Means of 4 Analyses, 5 Circles per Analysis. 0·15 ml. Applied to 2·3 sq.cm. Standard Errors.*

Concentration of solution (%)	DMBA applied (μg.)	DMBA in skin (μg.)
0·005	7·5	0·50 ± 0·07
0·01	15	0·81 ± 0·18
0·05	75	3·48 ± 0·09
0·10	150	2·44 ± 0·05
0·20	300	1·87 ± 0·03
0·30	400	1·38 ± 0·20
0·70	1050	1·55 ± 0·10
1·0	1500	1·88 ± 0·21

absorption of DMBA increased as the amount applied was increased from 7·5 to 75 μg., then decreased and became relatively constant when 300 to 1500 μg. was applied.

When an amount of 75 μg. DMBA was applied to the skin in 0·15 ml. of 0·05 per cent solution in acetone and the excess hydrocarbon was washed off at various times thereafter, the results shown in Table IV were obtained.

TABLE IV.—*The Penetration of DMBA into the Skin After the Application of 75 μg. (0·15 ml. of 0·05 per cent Solution in Acetone) to 2·3 sq.cm. Means of 3 Analyses, 5 Circles per Analysis. Standard Errors.*

Time (hours)	DMBA in skin (μg.)
0·25	0·15 ± 0·03
0·50	0·41 ± 0·05
1	0·89 ± 0·07
2	1·91 ± 0·13
3	3·48 ± 0·09
5	4·74 ± 0·15

Tumour incidence

The data for the distribution of tumours among all the mice exposed to DMBA are recorded in Table V. Also included in this table are the results obtained using a control group of mice dosed with acetone, immobilized for 7 hours and washed with diethyl ether. These mice, like those that had been treated with DMBA, were painted twice weekly with croton oil. Data for the time to appearance of the first tumour in a mouse (timed from the day that treatment with croton oil was begun) are recorded in Table VI. Times to appearance of all the tumours are reported in Table VII. A summary of all the data is given in Table VIII. Fig. 1 and 2 are discussed in the next section.

DISCUSSION

Before undertaking this work, we thought that variation in the amount of solid hydrocarbon applied to the skin, such as could be achieved by applying

TABLE V.—*Distribution of Tumours Among Mice*

Concentration DMBA (%)	Exposure (hours)	DMBA in skin (μg.)	Number of mice having following number of tumours																Number of mice	Number of tumours	Tumours per mouse	Standard deviation
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15				
Zero	—	—	83	6	1	..	..	..	..	..	..	..	..	..	..	..	..	..	90	8	0·09	..
0·65	0·25	0·15	22	14	9	6	1	..	2	..	..	1	..	..	..	..	..	..	55	75	1·37	1·78
75 μg. applied	0·50	0·41	26	8	6	7	4	1	3	1	..	..	1	..	..	..	..	..	57	97	1·70	2·22
	1	0·89	14	11	8	4	5	3	4	2	2	..	1	1	..	..	..	..	55	149	2·71	2·79
	3	3·48	19	9	8	4	7	4	2	1	1	2	..	1	..	..	..	..	58	141	2·43	2·69
	5	4·74	16	11	5	4	..	10	2	1	1	1	2	..	..	1	1	..	55	166	3·02	3·46
0·25	0·25	1·09	27	6	4	4	..	3	4	2	..	..	3	..	..	..	..	..	56	133	2·38	3·16
B 300 μg. applied	0·50	1·05	10	8	10	6	9	5	2	2	1	2	..	..	1	..	..	..	56	171	3·05	2·65
	1	1·40	18	8	5	3	6	1	4	1	2	1	1	..	..	..	..	..	50	122	2·44	2·78
	3	1·89	24	8	4	3	5	4	..	1	2	1	..	2	1	2	..	..	57	157	2·76	3·73
	5	2·09	24	9	9	5	3	2	..	..	2	..	..	2	..	1	1	..	58	129	2·23	3·33
0·25	0·25	0·35	29	9	5	4	3	1	2	..	2	1	..	..	..	..	..	..	56	85	1·52	2·29
H 100–150 μg. applied	0·50	0·35	16	15	9	3	5	2	2	..	2	..	..	..	..	..	..	..	54	100	1·86	2·04
	1	1·47	25	10	11	8	7	3	2	1	1	..	2	3	..	..	..	..	73	179	2·49	2·92
	3	2·73	27	8	9	6	12	4	6	1	1	..	..	..	..	..	..	..	74	163	2·24	2·17
	5	3·73	5	4	2	9	3	3	2	4	2	..	..	..	..	..	..	..	34	118	3·48	2·41
	7	4·18	6	3	4	5	5	8	3	2	2	..	..	..	..	..	..	1	39	149	3·80	2·96

TABLE VI.—*Time to Appearance of First Tumour in a Mouse Dated from Time Treatment with Croton Oil was Begun*

Concentration DMBA (%)	Exposure (hours)	DMBA in skin (μg.)	Number of mice bearing tumours in following number of weeks																	Total tumour bearers	Total mice
			3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
0·05	0·25	0·15	..	..	..	..	2	4	9	7	3	..	1	1	2	..	1	3	..	..	33	55
	0·50	0·41	..	..	..	..	2	5	8	5	3	1	2	3	1	..	1	..	..	..	31	57
	1·0	0·89	..	..	..	1	6	5	9	3	7	4	2	..	1	2	1	..	..	..	41	55
	3·0	3·48	..	..	..	..	4	2	9	8	1	4	1	7	1	1	1	..	..	..	39	58
	5·0	4·75	..	..	..	9	2	10	5	5	1	3	4	..	..	..	..	..	..	..	39	55
0·25 B	0·25	1·09	..	..	..	1	2	6	8	3	1	3	2	2	1	..	..	..	..	..	29	56
	0·50	1·05	..	..	..	.	4	9	15	7	2	6	1	1	..	..	..	1	..	..	46	56
	1·0	1·4	..	..	..	3	..	4	11	5	2	2	2	..	2	1	..	..	..	..	32	50
	3·0	1·89	..	..	..	1	4	4	7	4	4	6	2	..	..	..	..	..	..	1	33	57
	5·0	2·09	..	..	..	1	5	6	9	6	1	2	1	1	1	1	..	..	..	..	34	58
	7·0	..	..	1	..	1	5	4	3	3	1	2	..	1	1	..	..	2	..	..	24	33
0·25 H	0·25	0·35	..	..	7	1	4	4	2	3	1	2	1	1	1	..	..	..	..	..	27	56
	0·50	0·35	1	..	..	8	4	6	2	2	2	3	4	1	1	..	2	2	..	..	38	54
	1·0	1·47	..	..	..	1	6	8	8	4	8	2	2	2	1	3	1	..	..	..	46	73
	3·0	2·73	..	..	..	3	7	13	13	2	4	1	2	2	..	..	..	..	..	..	47	74
	5·0	3·73	..	..	..	..	5	10	6	1	2	4	..	..	..	..	..	1	..	..	29	34
	7·0	4·18	..	..	..	3	3	8	10	1	2	1	1	1	1	1	..	1	..	..	33	39

TABLE VII.—*Time to Appearance of All Tumours Dated from Time Treatment with Croton Oil was Begun*

Concentration DMBA (%)	Exposure (hours)	DMBA in skin (μg.)	Number of tumours recorded in following weeks																		Total tumours
			3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
0·05	0·25	0·15	..	..	..	..	3	8	11	14	7	3	3	5	3	7	7	4	..	..	75
	0·50	0·41	..	..	..	..	2	10	17	12	12	11	7	7	6	1	5	3	..	4	97
	1·0	0·89	..	..	..	1	9	7	28	16	16	25	11	8	9	11	4	1	2	1	149
	3·0	3·48	..	..	..	..	9	4	19	19	7	18	14	23	12	8	4	2	2	..	141
	5·0	4·74	..	..	..	17	19	28	16	37	11	21	7	3	2	5	..	..	..	..	166
0·25 B	0·25	1·09	..	..	..	1	6	18	30	24	9	10	12	4	3	8	2	3	1	2	133
	0·50	1·05	..	..	..	..	8	23	38	21	20	27	7	7	7	10	1	2	..	..	171
	1·0	1·40	..	..	..	6	3	9	25	22	9	10	8	4	12	7	3	3	..	1	122
	3·0	1·89	..	..	..	2	9	5	47	23	10	22	14	1	7	9	3	1	1	3	157
	5·0	2·09	..	..	..	1	7	10	37	17	15	17	6	9	3	3	1	2	..	1	129
	7·0	..	..	..	1	..	1	14	4	12	8	3	27	5	7	3	6	..	4	2	1	98
0·25 H	0·25	0·35	..	..	9	8	13	13	8	6	8	3	6	4	4	2	..	..	..	1	85
	0·50	0·35	1	..	..	9	11	12	12	7	6	6	12	4	7	5	3	5	..	..	100
	1·0	1·47	..	..	..	1	9	15	37	23	27	20	16	13	9	6	1	2	..	..	179
	3·0	2·73	..	..	..	3	13	25	42	25	14	15	5	8	7	2	2	2	..	..	163
	5·0	3·73	..	..	..	..	10	23	22	8	13	15	9	4	5	6	..	3	..	..	118
	7·0	4·18	..	..	..	4	18	24	24	6	20	12	5	19	6	4	4	3	..	..	149

TABLE VIII.—*Data of Tables II and IV to VII Rearranged to show Variation in Tumour Incidence and Latent Period with Variation in DMBA in Skin. Standard Errors are shown.*

DMBA in skin (μg.)	Tumours per mouse	Number of mice	Latent period	
			First tumour (weeks)	All tumours (weeks)
0·00	0·09	90	..	..
0·15 ± 0·03	1·36	55	10·85 ± 0·57	11·92 ± 0·39
0·35 ± 0·03	1·52	56	8·37 ± 0·57	9·29 ± 0·35
0·35 ± 0·08	1·86	54	9·84 ± 0·63	10·84 ± 0·34
0·41 ± 0·05	1·70	57	10·35 ± 0·45	11·80 ± 0·36
0·89 ± 0·07	2·71	55	10·09 ± 0·42	11·56 ± 0·40
1·05 ± 0·13	3·06	56	9·45 ± 0·31	10·78 ± 0·18
1·09 ± 0·05	2·38	56	9·86 ± 0·43	10·92 ± 0·26
1·40 ± 0·04	2·44	50	9·91 ± 0·44	11·23 ± 0·28
1·47 ± 0·13	2·49	73	10·32 ± 0·42	11·07 ± 0·21
1·89 ± 0·12	2·76	57	10·03 ± 0·46	11·08 ± 0·23
2·09 ± 0·06	2·23	58	9·89 ± 0·42	10·67 ± 0·23
2·73 ± 0·25	2·23	74	8·87 ± 0·29	11·05 ± 0·34
3·48 ± 0·09	2·43	58	10·85 ± 0·43	12·07 ± 0·24
3·73 ± 0·16	3·47	34	9·28 ± 0·44	10·61 ± 0·26
4·18 ± 0·06	3·82	39	9·48 ± 0·50	10·66 ± 0·17
4·74 ± 0·15	3·02	55	8·77 ± 0·36	11·05 ± 0·34

varying volumes or concentrations of a solution in a solvent that readily evaporates, should be without effect on the amount of the hydrocarbon absorbed by unit area in a given time, provided that enough was applied to maintain a saturated solution in the sebum. The latter condition should ensure the absorption of the hydrocarbon at the maximal rate. On this basis, the increased absorption of DMBA which resulted when the amount applied was increased from 7·5 to 75 μg. (Table III) may be explained by assuming that amounts less than 75 μg. were not enough to provide a saturated solution in the sebum. A different explanation has to be sought for the fact that the absorption decreased as the amount applied was increased from 75 to 300 μg. and thereafter became relatively constant. It seems probable that, as the bulk of the solid hydrocarbon was increased, it soaked up some of the sebum, particularly that readily available on the surface of the skin, as indeed any powdery substance should do. The importance of sebum for the absorption of DMBA was demonstrated in an experiment in which the skin was washed with diethyl ether before the hydrocarbon was applied. Under these conditions, only 0·56 μg. of DMBA was absorbed in 2 hours as compared with 1·91 μg. in the same time by unwashed skin.

The differences obtained by the two experimenters who measured the penetration of DMBA at various times (Table II) must be attributed to the fact that the quantities applied to the skin were different in the two experiments. One investigator (B) applied approximately 300 μg. or more; the other (H) applied only 100 to 150 μg., both in the belief (supported by our earlier experience with 3,4-benzopyrene—McCarter, 1956) that variation in the amount applied was without influence on the amount absorbed. The values recorded in Table II for 3 hours of penetration are consistent with predictions made on the basis of the amounts applied and the data of Table III. However, the data of Tables II and IV show that a greater *initial* penetration of DMBA took place when the amount applied was increased. Thus, in 0·25 hours, 0·15 μg. of 75, 0·35 of 100–150, and

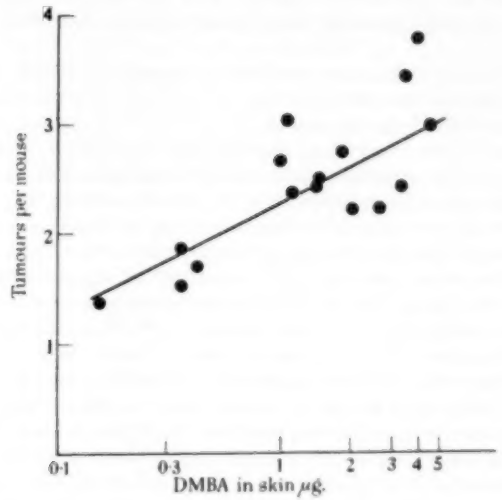

FIG. 1.—Tumours per mouse plotted against the logarithm of the amount of DMBA in the skin. The straight line fitted by the method of least squares has the equation $Y = 1 \cdot 13 + 1 \cdot 12 \log (10 \times \mu g.\ DMBA)$. Standard error of the slope = 0·21.

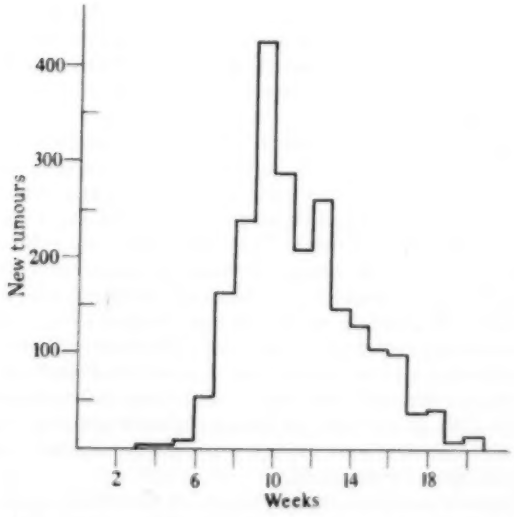

FIG. 2.—The number of new tumours observed in each week following the start of treatment with croton oil, calculated by summing the appropriate columns of Table VII.

1·09 of 300 or more had entered the skin. The factors governing the absorption of DMBA by mouse skin must be more complex than we have supposed them to be.

The data of Table IV were obtained having regard for all the known variables. The data are fitted by the straight line $Y = 0{\cdot}004 + 0{\cdot}96$ (hours of contact) where $Y = \mu$g. of DMBA per dosed circle (2·3 sq.cm.). The standard error of the slope was 0·01. The fact that the rate of absorption of the hydrocarbon was constant during the 5 hours' period under consideration suggests that there was no marked alteration in permeability of the skin during that time.

The mistake made in assuming that variation in the amount applied would have no influence on the amount absorbed, would have made it impossible to compare the data on tumour incidence obtained by the two investigators, and certainly it would not have been possible to combine the results as has been done in Table VIII, but for the fact that the amount of DMBA in the skin was measured, thus providing a common basis for comparison. It was important to know that each set of experiments had been performed with a high degree of reproducibility (standard errors, Table III) so that reliance could be placed in the corresponding analytical and tumour data. It was possible, therefore, to use all of the data.

The information provided by these experiments was examined for the existence of relationships between the amount of hydrocarbon in the skin (disregarding the time for absorption as a factor) and the tumour yield. For each group that had received a given dose two measures were available ; one was the average number of tumours borne by the animals that survived the experiment : the other was the proportion of tumour-bearing mice relative to the number of survivors. It must be stressed that the number of tumours recorded per mouse was the number of new tumours produced during the whole period of 20 weeks and was not the incidence at any particular time. The index we have used is independent of the balance struck between the rate of appearance of new tumours and the rate of disappearance of old (discussed by Salaman, 1958). We have not tried to do any calculations relating the rate of regression of tumours to the dose of DMBA (the amount in the skin) because we are uncertain about how to score a tumour as one regressing or having regressed.

The amount of hydrocarbon found in the skin by analysis is less than that which has penetrated because some is metabolized or transported from the site. The rate of loss of DMBA left after washing off the excess and measured over the first twelve hour period following absorption, has a half-life time of the order of 10 hours (Huh and McCarter, 1960). This value may be used to estimate, for example, that during the 7 hour period at the end of which 4·74 μg. DMBA was present in the skin, 6·2 must have been absorbed, of which 1·4 had been metabolized or transported. This value is very much smaller than that estimated by Booth and Boutwell (1959) who attributed the difference between the amounts of DMBA applied to the skin and recovered later at the site of application in restrained animals, to absorption through the skin. The rates of accumulation and disappearance measured by us are not consistent with the assumption made by these authors. The correction for loss during absorption was not applied by us in assessing the dose-response relationships.

The data of Table VI were analyzed using the method of probits as described by Finney (1950). There was no evidence that the results could not be described by a straight line having the equation $Y = 5{\cdot}17 + 0{\cdot}18 \log (10 \times \mu$g. DMBA in skin)

where Y = probits of response. The variance of the slope was 0·11 ; thus it could not be decided whether or not there was a dependence of tumour yield on log dose.

The data of Table VIII show that an increase in the amount of DMBA from 0 to 0·15 ± 0·03 μg. was accompanied by an increase in the number of tumours produced per mouse by a factor of 15, whereas a further increase to 4·74 ± 0·15 μg. (factor of 30, or factor of 40 if the value is corrected for metabolic loss) only doubled the tumour yield. An analysis of variance of the data for the effect of variation in log dose on the number of tumours produced per mouse was carried out according to Snedecor (1946). There was a highly significant ($P < 0{\cdot}001$) regression of tumour yield on the logarithm of the amount of DMBA in the skin. The experimental results are described by the equation Tumours per mouse = 1·13 + 1·12 log (10 × μg. DMBA in skin). The standard error of the slope was 0·21. It would be incorrect to assume either that this relationship is the only one capable of describing the data or that it can be used to extrapolate much beyond the limits of the data. One cannot decide whether or not the tumour yield continues to increase or if it approaches some limiting value as the amount of DMBA is extrapolated to higher values, though it seems apparent that the response is virtually saturated for amounts of DMBA in the skin greater than 1 or 2 μg. per dosed circle (about 0·5 to 1 μg. per sq.cm.). We have no explanation to offer for this apparent approach to saturation unless, perhaps, this observation represents the early onset of the phenomenon recorded by Shubik and Ritchie (1953) as a failure to obtain summation of effects of multiple doses of DMBA. Similarly, it is not possible to decide if there is, or is not, a threshold dose for the initiation of tumours, though it seems clear that if such a dose exists it must be very small indeed.

If the data of the groups (rows) of Table V are plotted with numbers of animals as ordinates and numbers of tumours borne by them (0, 1, 2, 3, etc.) as abscissae, it is learned that the distributions are J-shaped. It might have been expected that the data should conform to a Poisson distribution, but there are very significant deviations of the observed values from the expected, particularly for animals having no tumours and for those having many tumours. In both instances, the numbers observed far exceed the numbers predicted. We attempted to analyze our data by the methods described by Polissar and Shimkin (1954) but without conclusive result. The range of responses observed by us (1·36 tumours per mouse with the lowest dose and 3·02 with the highest) was too narrow to permit a trend to be seen when the standard deviations were plotted against the means. For every group, the observed standard deviation was greater than the calculated (S.D. = $m^{\frac{1}{2}}$ for a Poisson) but it was not possible to obtain evidence for or against the alternative hypotheses that the deviations from a Poisson distribution were due to *A*, heterogeneity of susceptibility among the animals, or *B*, interaction between the tumours. The CFW mouse is not inbred, so that hypothesis *A* seems a logical explanation of our results. On the other hand, it is conceivable, in the present state of our ignorance, that conditions that allow one tumour to appear in a mouse, might allow other tumours to appear with a greater frequency than would be predicted on a random basis.

If DMBA applied to the skin is left on, instead of being washed off as in our experiments, a single dose may lead to epilation and necrosis of the treated skin (Orr, 1938) and may result in the production of tumours without further treatment (Englebreth-Holm and Iversen, 1951). In our experiments, 31 CFW female mice

that had, initially, 4·2 μg. of DMBA in the skin failed to develop any tumours in the subsequent 20 weeks' period during which they were not treated with croton oil. We have not observed in our animals any loss of hair at the treated site in the three weeks' interval between the time of the limited exposure to DMBA and the start of treatment with croton oil. On the other hand, when we left the hydrocarbon on the skin, epilation and necrosis were always observed. More detailed investigation is needed, but these observations suggest that initiation of tumours by DMBA may be achieved with a minute amount of hydrocarbon that does not produce apparent injury to the skin; injury results from the penetration of a larger quantity of the hydrocarbon, but the number of tumours initiated is not thereby necessarily increased. This is not to imply that injury is not important in the overall process of tumour production, but that for DMBA applied to skin, as for urethane (Roe and Salaman, 1954) and triethylene melamine (Roe and Salaman, 1955) and for DMBA by mouth (Berenblum and Haran-Ghera, 1957) initiation of skin tumours may be dissociated from injury.

Altogether, 2510 tumours of the skin were recorded in this study. Judged by their appearance, growth-rate and invasiveness (or lack of it), only 2 were malignant and the remainder were papillomas. Of 655 tumours in 280 mice treated with 75 μg. DMBA applied to 2·3 sq.cm. only 18 (2·7 per cent) arose outside this area. These were situated on the flanks or near the base of the tail but were always close to the treated circle. Contamination of the skin around this area with a minute amount of DMBA during the washing procedure could account for the distribution of these tumours. The chance of contaminating the skin was greater when the applied quantity of DMBA was greater. Of 1855 tumours produced in 638 mice dosed with 100 to 300 μg. 235, or 12 per cent arose outside, but near, the treated area. Tumours that were obviously outside the treated areas were neglected in compiling Tables V to VIII.

A possible source of error in these experiments could arise from tumours growing close together so that they might be indistinguishable from one another. Tumours were first recognised when they had an area of approximately 1 sq.mm. or about 0·5 per cent of the area in which they could appear. Even in the groups having the highest tumour incidence, tumour-bearing animals had an average of 4·3 each, with a maximum of 15 so that the area occupied when the tumours were first recognisable was a negligible proportion of the total available. Growth of the early-appearing tumours might have interfered with the recording of those appearing late but this could only have been a problem in the animals bearing a large number of tumours, for example, 10 or more. Such animals accounted for only 14 per cent of all tumours produced. Furthermore, most of the tumours (80 per cent) appeared within a period of time (7th to 13th weeks) when all were small and least likely to overgrow one another. It is not likely, therefore, that inability to distinguish tumours was a major source of error in these experiments.

The latent period, dated from the time treatment with croton oil was begun to the time of appearance of the first tumour in a mouse, or averaged for all the tumours appearing in a mouse, did not change in a systematic way with variation in the amount of DMBA in the skin. Inspection of Table VII and Fig. 2 might suggest that groups of tumours arose characterized by different latent periods, indicating perhaps that the tumours were of more than one sort, and lending support to the suggestion made by Shubik, Baserga and Ritchie (1953) that tumours produced by initiating and promoting stimuli in mouse skin differ over

a range of growth potential. There is, however, no reason to suppose that the fluctuations we have observed are other than random variations due to chance. Perhaps real differences would have been observed had the animals been kept for a longer time, but we decided to stop the experiment when, as shown in Fig. 2, the tumours stopped appearing.

The methods described in this paper should be capable of furnishing estimates of the relative initiating potencies of different hydrocarbons. We have found by analysis that a unit area of CFW mouse skin absorbs in 1 hour $1{\cdot}4 \times 10^{-9}$ moles of 3,4-benzopyrene and $5{\cdot}5 \times 10^{-9}$ moles of DMBA, after application of acetone solutions of the hydrocarbons. The corresponding tumour incidences were 0·3 tumours per mouse for 3,4-benzopyrene and 2·44 per mouse for DMBA. Was the number induced by DMBA greater than that induced by 3,4-benzopyrene merely because there was more DMBA in the skin? The question cannot be answered without knowing more about the dose-response relationships for the two hydrocarbons.

Earlier work with 3,4-benzopyrene (McCarter, 1958) in mice of the strain I, suggested a pattern of dose and response similar to that recorded here. An attempt to use 3,4-benzopyrene in the CFW mouse resulted in the production of too few tumours to allow conclusions to be drawn with confidence, but the data are approximately described by the equation, Tumours per mouse $= 0{\cdot}25 + 0{\cdot}04$ (moles $\times 10^{-9}$ BP/sq.cm.). The rate of penetration of benzopyrene into CFW skin did not differ significantly from that in I skin. These data allow a rough estimate to be made of the relative sensitivities of the two strains of mouse. For example, 2×10^{-9} moles of 3,4-benzopyrene per sq.cm. produced 1·8 tumours per mouse in strain I compared with 0·3 in strain CFW. The same dose of DMBA produced 1·9 tumours per CFW mouse. Apparently, the strain I mouse is about 6 times as sensitive as the CFW, and DMBA is 6 or 7 times as potent an initiating agent as 3,4-benzopyrene. However, these estimates are only approximate because in the CFW mouse, the only parts of the dose-response curves for the two hydrocarbons that overlap involve the highest doses of 3,4-bezopyrene and the lowest of DMBA ; no point was obtained at which the tumour yields were identical and it is not possible to decide if the ratio of responses is independent of dose. One would wish to compare the curves over the regions where response is most markedly dependent on dose rather than where virtual saturation of the biological effect is achieved but this would involve one in the measurement of smaller doses and responses with larger errors than were encountered in the present work. Had the present experiments been done on a smaller scale or with less rigorous control of the variables concerned, it is probable that we should not have been able to draw even the limited conclusions that we have drawn about the nature of the dose-response relationships.

SUMMARY

1. 9,10-Dimethyl-1,2-benzanthracene (DMBA) was applied in acetone to 2·3 sq.cm. of the skin of immobilized mice. After a time, the skin was washed and some of the treated animals were killed for analysis of the DMBA content of the skin ; others were allowed to live and were treated topically with croton oil to produce tumours.

2. The amount of DMBA entering the skin was dependent on the amount applied and on the time allowed for absorption.

3. The number of tumours borne per mouse was a linear function of the logarithm of the amount of DMBA in the skin.

4. Animals bearing no tumours, and those bearing many, were more numerous than predicted by a Poisson distribution.

5. The time from the start of treatment with croton oil to the appearance of the first tumour, or averaged for all the tumours in a group, appeared to be independent of the amount of hydrocarbon in the skin.

6. Difficulties in the way of obtaining relative potency ratios of different hydrocarbons are discussed.

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PHENANTHRENE AS AN ANTI-INITIATING AGENT

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In 1945, Lacassagne, Buu-Hoi and Rudali reported that several weakly carcinogenic substances when painted on mouse skin alternately with 20-methylcholanthrene reduced the ability of the latter substance to produce tumours. This anti-carcinogenic effect was attributed to competition between the weak and strong carcinogens for some substrate within the cell because of their analogous structure.

In the following year, Crabtree (1946) showed that the non-carcinogenic hydrocarbons, anthracene, naphthalene and especially phenanthrene possessed anti-carcinogenic activity when tested in multiple applications against 3,4-benzopyrene and 20-methylcholanthrene on the skin of mice. Investigations reported by Riegel *et al.* (1951) using 20-methylcholanthrene and by Hill *et al.* (1951) using 9,10-dimethyl-1,2-benzanthracene showed that several hydrocarbons depressed carcinogenic potency but others enhanced it.

In all of the above studies multiple applications of the hydrocarbons were used, thus making it difficult to decide at what stage in the process of tumour production the effect of the anti-carcinogenic agent was exerted. We tried to simplify the experiment by testing the effect of a single application of phenanthrene on the yield of tumours induced by a single application of 9,10-dimethyl-1,2-benzanthracene (DMBA) and multiple applications of croton oil. Some of the methods used have been described previously (McCarter, 1956; McCarter, Szerb and Thompson, 1956; Ball and McCarter, 1960).

EXPERIMENTAL

The hydrocarbons 9,10-dimethyl-1,2-benzanthracene and phenanthrene (Eastman Kodak Co., Rochester, New York) were purified by chromatography on Florisil (60-100 mesh, Floridin Co., Tallahasee, Florida) and were checked for purity by measurement of the ultraviolet absorption spectra. Solutions were prepared by dissolving the substance in acetone to make mixtures having the compositions shown in Table I.

All solvents were distilled before use. Cyclohexane was further purified by passing it through a column of silica gel (28–200 mesh, Fisher Scientific Co., Montreal, Canada).

The animals used were female mice of the CFW strain 8 to 10 weeks of age. The procedures used in the care of the animals, for the application of hydrocarbons and removal of excess from the skin, and for the production of tumours by multiple applications of croton oil have been described in the preceding paper (Ball and McCarter, 1960). As in those experiments, so in these, some animals were kept for the production of tumours; others were killed immediately so that the dosed

TABLE I.—*Penetration and Persistence of DMBA and Phenanthrene in Skin of CFW Mice. 0·15 ml. of Acetone Solution Applied to Circle 1·54 to 1·66 sq.cm. on Back. Each Result Derived from 3 Analyses, 10 Treated Circles per Analysis. Standard Deviation.*

Solution applied (%)	Hydrocarbon in skin at following times after application (μg. per sq. cm.)						
	0·5 hour	1 hour	3 hours	6 hours	9 hours	15 hours	27 hours
DMBA 0·2	0·16 ± 0·08	0·65 ± 0·13	1·21 ± 0·15	0·87 ± 0·05	0·70 ± 0·07	0·35 ± 0·04	0·0
Phenanthrene —	..	..	..	..	..	..	..
DMBA 0·2	0·49 ± 0·05	1·12 ± 0·07	2·27 ± 0·41	1·38 ± 0·05	1·16 ± 0·1	0·34 ± 0·05	0·0
Phenanthrene 0·05	1·06 ± 0·25	1·66 ± 0·18	3·05 ± 0·55	1·06 ± 0·14	0·52 ± 0·04	0·0	0·0
DMBA 0·2	0·82 ± 0·05	1·05 ± 0·06	1·68 ± 0·33	1·57 ± 0·14	1·09 ± 0·18	0·91 ± 0·05	0·0
Phenanthrene 0·1	0·62 ± 0·04	0·87 ± 0·37	3·39 ± 0·46	1·20 ± 0·28	0·67 ± 0·21	0·0	0·0
DMBA 0·2	0·75 ± 0·09	0·94 ± 0·09	0·87 ± 0·17	0·78 ± 0·33	0·47 ± 0·04	0·0	0·0
Phenanthrene 0·2	1·24 ± 0·07	1·49 ± 0·18	3·22 ± 0·19	1·66 ± 0·26	0·72 ± 0·1	0·0	0·0
DMBA —	..	..	..	..	..	..	..
Phenanthrene 0·2	1·29 ± 0·22	1·89 ± 0·38	6·42 ± 0·17	2·44 ± 0·26	0·68 ± 0·05	0·0	0·0

Excess hydrocarbon washed off skin after 3 hours.

circle of skin could be excised and analyzed for its content of hydrocarbons. In order to measure the persistence of the hydrocarbons once they had penetrated the skin, still other animals were killed at intervals up to 24 hours after washing off the excess of hydrocarbon and the dosed circles of skin were then taken for analysis. Pertinent details will be found in Table I.

Analytical method

Ten dosed circles of skin obtained from 5 mice were weighed together and were then placed in a Soxhlet apparatus where they were extracted for 48 hours with approximately 80 ml. of 95 per cent ethanol. To the extract, 2 ml. of 50 per cent potassium hydroxide was added and the mixture was heated under a reflux condenser for 4 hours to saponify lipids. The mixture was then cooled, diluted with 40 ml. of water and was repeatedly extracted with cyclohexane. For this purpose, it was found convenient to divide the mixture into two parts and to extract each separately in a glass U-tube using 10 ml. volumes of cyclohexane four times. The pooled extracts were then transferred to a column of Florisil, 60–100 mesh, 35 g. 2·2 cm. in diameter and 14 to 15 cm. long, for chromatographic separation of the two hydrocarbons.

Phenanthrene was contained in the fractions collected between 80 and 180 ml. and was completely eluted from the column by cyclohexane. Preliminary tests had shown that DMBA did not appear until 220 ml. had run through the column and it could not be completely removed by cyclohexane. Therefore, as soon as the phenanthrene had been collected, the addition of cyclohexane to the column was stopped and a mixture was substituted consisting of benzene and petroleum ether (b.p. 40–60° C.) in the proportions 20 : 80 (v : v). The removal of DMBA from the column began at once and was complete when approximately 100 ml. of eluate had been collected.

Because of the volatility of phenanthrene, the solvent could not be evaporated ; hence no reduction in volume could readily be achieved. The fractions containing phenanthrene were, therefore, pooled in a 150 ml. volumetric flask.

The fractions containing DMBA were pooled and evaporated to dryness under reduced pressure. The residue was dissolved in 95 per cent ethanol and the volume was adjusted to 50 ml.

The absorption spectra of the solutions were measured relative to those of control preparations derived from mouse skin dosed with acetone alone. Measurements were made over the range 220 to 320 mμ. using silica cells having a light path of 1 cm. in the Beckman DK-2 Ratio Recording Spectrophotometer. The concentration of phenanthrene was calculated from the molar extinction coefficient $E_{molar} = 4{\cdot}83$ in cyclohexane at 252 mμ. and of DMBA from $E_{molar} = 4{\cdot}90$ in ethanol at 297 mμ.

In experiments to test the efficiency of the analytical procedure, 20 μg. of phenanthrene and 20 to 60 μg. of DMBA added to mouse skin were accounted for to the extent of 98 and 99 per cent respectively.

DISCUSSION

The possibility existed that admixture of phenanthrene with DMBA might reduce the absorption of the latter by the skin with a concomitant reduction in the tumour yield. We observed, however, that the absorption of DMBA was actually increased by mixing the two hydrocarbons in the ratios 1 : 4 and 1 : 2 respectively (Table I). It was only when the ratio was 1 : 1 that the absorption of DMBA was reduced.

The data of Table II show that the tumour yields induced by the mixtures of phenanthrene and DMBA were lower than the number obtained using DMBA alone. This is particularly evident for the results showing that 1·21 μg. of DMBA per sq.cm. of skin in the absence of phenanthrene produced 2·2 tumours per mouse whereas, slightly more DMBA (1·68 μg.) in the presence of 3·39 μg. of phenanthrene produced only 0·62 tumours per mouse. The difference is significant at $P = 0{\cdot}01$.

It is unfortunate that the experiment was terminated at the end of the 20 weeks' period of croton oil treatment. It is evident from Table III that the production of new tumours in the group treated with DMBA alone had stopped, but in the groups treated with mixtures of the two hydrocarbons, new tumours were still slowly appearing. Not only was the rate of appearance of the tumours slowed, but the rates of growth seemed also to be slowed. None of the tumours induced by the mixtures grew quickly, or large, in sharp contrast to the behaviour of tumours induced by DMBA without phenanthrene. All the tumours were papillomas.

There can be no doubt that phenanthrene modified the initiating effect of DMBA, but whether this consisted in a real decrease in the number of tumours that could have been produced, or an apparent decrease due to a delayed rate of appearance it is not possible to say on the present evidence.

A tentative inquiry can be made of the magnitude of the effect produced by phenanthrene. If the dose response relationship, tumours per mouse $= 1{\cdot}13 + 1{\cdot}12 \log (10 \times \mu g.\ DMBA)$ established by Ball and McCarter (1960) is taken to apply to the present experiment (the conditions used were very similar) it may be

TABLE II.—*Distribution of Tumours Elicited by 2·5 per cent Croton Oil in Liquid Paraffin Applied Twice Weekly to CFW Female Mice Treated Once on the Back over an Area of 1·54 to 1·66 sq.cm. with DMBA or Phenanthrene, or Mixtures of the Two Hydrocarbons. Excess of the Applied Agent Washed Off Skin Using Diethyl Ether 3 Hours after Application. Solvent for Hydrocarbons was Acetone. Interval between Initiating and Promoting Treatment was 3 Weeks.*

Solution applied	Number surviving mice	Number of mice bearing following number of tumours											Number of tumours	Tumours per mouse	Amount hydrocarbon in skin μg. sq. cm.
		0	1	2	3	4	5	6	7	8	9	10			
Acetone	90	83	6	1	—	—	—	—	—	—	—	—	8	0·09	—
DMBA 0·2% Phenanthrene —	74	27	8	9	6	12	4	6	1	1	—	—	163	2·2	1·21 —
DMBA 0·2% Phenanthrene 0·05%	36	19	6	5	1	1	—	2	—	1	—	1	53	1·47	2·27 3·05
DMBA 0·2% Phenanthrene 0·1%	35	24	4	4	2	1	—	—	—	—	—	—	22	0·62	1·68 3·39
DMBA 0·2% Phenanthrene 0·2%	40	24	8	1	1	4	2	—	—	—	—	—	39	0·97	0·87 3·32
DMBA — Phenanthrene 0·2%	39	38	1	—	—	—	—	—	—	—	—	—	1	0·03	— 6·42

calculated that 0·62 tumours would be produced by 0·04 μg. of DMBA. This result would imply that in this experiment, phenanthrene counteracted the initiating activity of 97 per cent of the DMBA that was in the skin. Perhaps the inhibition might have been complete had the phenanthrene persisted longer in the skin, but the data of Table I show that it disappears at a somewhat faster rate than DMBA. The latter substance might, therefore have been free to act though in small quantity and for a short time, in the absence of phenanthrene.

We are extending this work by applying the substances at different times in relation to one another. A point of possible practical interest is that phenanthrene

TABLE III.—*Latent Period of all Tumours Observed*

Solution applied	Number of new tumours observed in following number of weeks														
	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
DMBA 0·2% Phenanthrene —	3	13	25	42	25	14	15	5	8	7	2	2	2	0	0
DMBA 0·2% Phenanthrene 0·05%	2	4	5	18	0	0	3	2	0	5	2	2	1	9	0
DMBA 0·2% Phenanthrene 0·1%	1	2	0	1	1	4	3	1	1	1	1	3	2	1	
DMBA 0·2% Phenanthrene 0·2%	1	2	1	4	4	6	3	2	1	2	3	0	3	3	4
DMBA — Phenanthrene 0·2%	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—

is known to be a prominent constituent of tobacco tar (Campbell and Lindsey, 1956) and the possibility exists that its presence there modifies the carcinogenic activity of the tar.

This work was supported by a grant from the National Cancer Institute of Canada.

SUMMARY

The absorption and persistence of 9,10-dimethyl-1,2-benzanthracene (DMBA) and phenanthrene in mouse skin were measured following application of a mixture of the two hydrocarbons.

When the proportions of the former to the latter were 4 : 1 or 2 : 1 the amount of DMBA entering the skin was greater than when DMBA was applied alone, but the rate of appearance of tumours elicited by repeated treatment of the skin with croton oil was reduced. It is suggested that phenanthrene inhibits the initiating activity of DMBA.

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SURVEY OF PAPERS

Stocks finds that lung cancer mortality is strongly correlated with smoke density in the atmosphere in a number of different areas of England and Wales, these relations being only partially explicable by social differences in the populations concerned. Bronchitis and pneumonia in males and bronchitis also in females show similar strong correlations with smoke (p. 397).

Staszewski finds a distinct correlation between tobacco smoking and cancer of the lip, oral cavity, tonsils and larynx in men in Poland (p. 419).

De Waard, de Laive and Baanders-Van Halewijn find two peaks in the age distribution of mammary cancer at about 48 years and 65 years. They discuss the hypothesis that relates the former to ovarian, and the latter to adrenal dysfunction (p. 437).

Herdan reports an investigation into the association of malignant disease and diabetes. He finds that the observed overall negative association between the two diseases is true only for the female sex, and discusses possible explanations for this based on the disturbance of glucose metabolism in both diseases (p. 449).

Patey, from the study of a small series of patients, reaches the interim conclusion that oophorectomy and adrenalectomy, performed as soon as possible after the primary removal of a carcinoma of the breast by standard surgical methods, are unlikely to offer a significant contribution to the problem of carcinoma of the breast (p. 457).

Sim, Hobkirk, Stewart, Blair and Forrest have estimated the urinary 17-ketosteroids and their fractions in women with breast cancer before and after adrenalectomy and oophorectomy and implantation of the pituitary gland with yttrium–90. They find no relationship between pre-operative levels and the response to endocrine surgery. The findings in the urine after pituitary implantation with yttrium–90 indicate that full adrenal suppression is not required for benefit from this operation (p. 460).

Whitaker finds a highly significant difference between the plasma β-glucuronidase levels of 47 patients with breast cancer and 50 normal women (p. 471).

Heath finds that the injection of cobalt metal powder into the thigh muscles of rats often results in breakdown of the differentiated muscle fibres into free myoblasts, with the transformation of some of these myoblasts into malignant variants and the formation of characteristic malignant tumours (p. 478).

Illman and Ghadially have repeatedly painted the skin of hamsters with DMBA. They find that many melanotic tumours arise in brown and white hamsters, but none in the cream variety. The tumours in the brown animals contain abundant melanin, but those in the white hamsters are usually amelanotic. They discuss the significance of these findings (p. 483).

Cherry and Glucksmann find that ovariectomy reduces the incidence of vaginal tumours in rats after intravaginal application of DMBA, and administration of oestrogen or of progesterone raises the incidence only slightly. Adrenalectomy or repeated pelvic irradiation restores the level of tumour incidence in these ovariectomised animals to that seen in intact and pregnant rats (p. 489).

Pullinger finds that subjecting the mice to late ovariectomy results in a 7 to 13 fold reduction in the incidence of mammary carcinoma in former C_3Hf breeders (p. 502).

RANADIVE, HAKIM and KHARKAR find that pseudopregnancy in mice leads to a very high incidence of mammary tumours following skin painting with 20-methylcholanthrene, confirming the importance of luteal factor as a promoting agent in chemical carcinogenesis (p. 508).

MARCHANT reports treating young adult virgin mice with DMBA by skin painting, and later either reciprocally exchanging their ovaries with those of untreated mice, or exchanging only one ovary and re-implanting the other. She finds that the presence of grafts of normal ovaries inhibits the development of ovarian tumours in mice bearing unilateral grafts from animals pretreated with DMBA (p. 514).

MARCHANT finds that a high proportion of mice bearing bilateral grafts of ovaries from DMBA-treated mice develop ovarian tumours. The proportion of mice bearing tumours does not vary greatly with durations of pretreatment of the ovarian grafts from 10 to 60 days. No tumours develop in mice grafted with normal ovaries (p. 519).

DARCY reports a quantitative study of the rat serum α-globulin previously found to be associated with tissue growth. The protein reaches a high level in foetal and young rats and in pregnant females, compared with the normal adult level (p. 524).

DARCY compares the levels of a rat serum α-globulin reached during tumour growth with the levels in normal animals found in the preceding paper. The protein does not originate at the site of the tumour, but seems to be selectively absorbed from the surrounding fluid by the tumour (p. 534).

SAHASRABUDHE, NERURKAR, NARURKAR, TILAK and BHAVSAR report on the tumour inhibiting properties of thiophene 2 : 5-dicarboxylic acid, a substance which interferes with the hexose-mono-phosphate pathway. They find significant inhibition of growth of a fibrosarcoma in Swiss mice and increased survival of Yoshida sarcoma bearing rats (p. 547).

THOMLINSON describes a technique for growing tumours in the subcutaneous tissue of the rat in such a way that they remain localised until they reach a size suitable for testing effects of different treatments. The effects of oxygenation on the response of such a tumour to radiation are reported (p. 555).

BALL and MCCARTER report attempts to relate the skin tumour incidence in mice after croton oil treatment to the amount of initiating agent, DMBA, that penetrates unit area of skin in a given time (p. 577).

HUH and MCCARTER produce evidence suggesting that when a mixture of DMBA and phenanthrene is applied to the skin of mice as an initiating agent the presence of the latter inhibits the activity of the former (p. 591).

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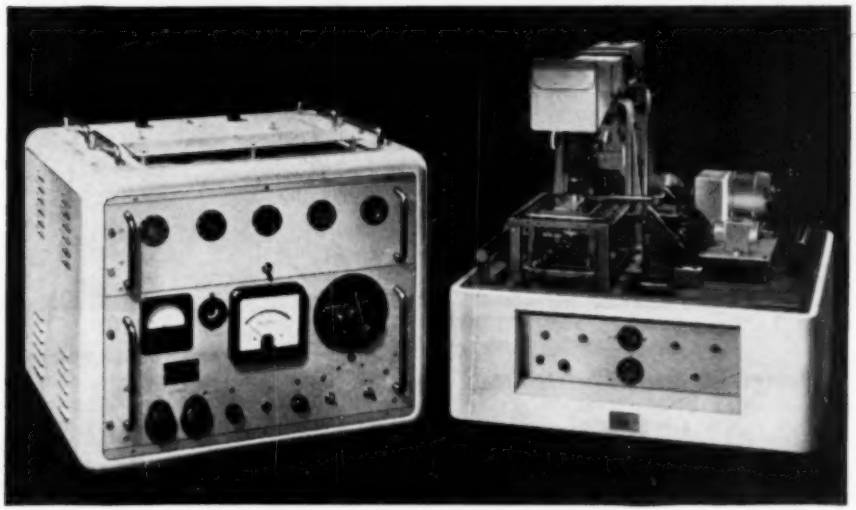

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